UNITED STATES DISTRICT COURT
EASTERN DISTRICT OF WISCONSIN

EMD CROP BIOSCIENCE INC., and EMD CROP BIOSCIENCE CANADA INC. Plaintiffs	:	
V.	:	Case No. _____
BECKER UNDERWOOD, INC., Defendant.	:	

COMPLAINT FOR PATENT INFRINGEMENT

Plaintiffs, EMD Crop Bioscience Inc. and EMD Crop Bioscience Canada Inc. (collectively "EMD"), by its attorneys, Godfrey & Kahn, S.C., and RatnerPrestia, alleges its complaint against Defendant, Becker Underwood, Inc. ("Becker Underwood") as follows:

THE PARTIES

1. Plaintiff EMD Crop Bioscience, Inc. is a corporation organized and existing under the law of the state of Delaware, with its principal place of business at 13100 West Lisbon Avenue, Suite 600, Brookfield, WI 53005.

2. Plaintiff EMD Crop Bioscience Canada Inc is a corporation organized and existing under the laws of Ontario, Canada, with its principal place of business at 145 Turnbull Ct., Cambridge, On, N1T 1C6, Canada.

3. Upon information and belief, Defendant Becker Underwood is incorporated under the laws of the state of Delaware, with its principal place of business at 801 Dayton Avenue, Ames, Iowa 50010.

JURISDICTION & VENUE

4. This action arises under the patent laws of the United States, Title 35 U.S.C. § 1 *et. seq.*

5. This Court has exclusive subject matter jurisdiction under 28 U.S.C. § 1338(a).

6. Venue is proper in the Eastern District of Wisconsin under 28 U.S.C. §§ 1391 and 1400.

7. This Court has personal jurisdiction over the defendant pursuant to Wis. Stat. § 801.05(1), (3) and (4), because: Becker Underwood is engaged in substantial and not isolated activities in State and this judicial district; Becker Underwood has offered to sell and/or sold infringing products in this State and this judicial district; and EMD has suffered injury within this judicial district, solicitation activities have been carried out in this judicial district by or on behalf of Becker Underwood, and products made by Becker Underwood will be used within this judicial district in the ordinary course of trade.

BACKGROUND

EMD and The EMD Patents

8. EMD is one of the world's leading developers of crop-enhancing products. EMD has marketed its Optimize® product line, including the Optimize® 400 product for soybeans, that contains the LCO (Lipo-chitooligosaccharide) Promoter Technology®. EMD is the licensee of several patents covering the LCO Promoter Technology® in the Optimize® 400 product.

9. Centre National de law Recherche Scientifique (C.N.R.S.) and Institut National de law Recherche Agronomique (I.N.R.A.) are the owners of all right, title, and interest in and to U.S. Patent No. 5,646,018 (the "'018 Patent") entitled "Broad Host Spectrum Rhizobiaceae Nodulation Signals." The '018 Patent was duly issued by the United States Patent & Trademark Office on July 8, 1997, and is valid and enforceable. A copy of the '018 Patent is attached hereto as Exhibit A.

10. Centre National de law Recherche Scientifique (C.N.R.S.) and Institut National de law Recherche Agronomique (I.N.R.A.) are the owners of all right, title, and interest in and to U.S. Patent No. 5,549,718 (the "'718 Patent") entitled "Substance with Lipo-Oligosaccharide Structure Capable of Acting as Plant-Specific Symbiotic Signals, Processes for Producing Them and Their Applications." The '718 Patent was duly issued by the United States Patent & Trademark Office on August 27, 1996, and is valid and enforceable. A copy of the '718 Patent is attached hereto as Exhibit B.

11. EMD has been granted an exclusive license under the '018 Patent and the '718 Patent.

12. EMD Crop Bioscience Canada, Inc. and McGill University are the owners of all right, title, and interest in and to U.S. Patent No. 6,979,664 (the "'664 Patent") entitled "Composition for Accelerating Seed Germination and Plant Growth." The '664 Patent was duly issued by the United States Patent & Trademark Office on December 27, 2005, and is valid and enforceable. A copy of the '664 Patent is attached hereto as Exhibit C.

13. McGill University has exclusively licensed its rights under the '664 Patent to EMD.

14. The University of Tennessee Research Foundation ("UTRF") is the co-owner of all right, title, and interest in and to U.S. Patent No. 5,175,149 (the "'149 Patent") entitled "Pentasaccharide Phytohormones and Methods for Their Use." The '149 Patent was duly issued by the United States Patent & Trademark Office on December 29, 1992, and is valid and enforceable. A copy of the '149 Patent is attached hereto as Exhibit D.

15. The UTRF is the co-owner of all right, title, and interest in and to U.S. Patent No. 5,321,011 (the "'011 Patent") entitled "Pentasaccharide Phytohormones and Methods for Their Use." The '011 Patent was duly issued by the United States Patent & Trademark Office on June 14, 1994, and is valid and enforceable. A copy of the '011 Patent is attached hereto as Exhibit E.

16. The UTRF has granted to EMD an exclusive license under the '149 Patent and the '011 Patent.

17. Patents covering the LCO Promoter Technology® in the Optimize® 400 product include the '018 Patent, the '718 Patent, the '664 Patent, the '149 Patent, and the '011 Patent.

Becker Underwood and its Infringement

18. Upon information and belief, Becker Underwood has used, offered for sale, marketed and/or sold a product called VAULT® HP as a growth enhancement system for soybeans. Becker Underwood has marketed the VAULT® HP product as an "ultra-low volume, high performance growth enhancement system for soybeans" containing a *Rhizobial* Inoculant.

19. Upon information and belief, Becker Underwood sells, offers to sell and/or advertises its VAULT® HP product in the United States, including the Eastern District of Wisconsin.

20. Upon information and belief, Becker Underwood's VAULT® HP product encompasses technology disclosed to and licensed to it by McGill, including technology described in U.S. Patent No. 7,262,151 (the "'151 Patent") entitled, "Methods and Compositions for Production of Lipo-Chito Oligosaccaharides by Rhizobacteria." A copy of the '151 Patent is attached as Exhibit F.

21. On March 24, 2006, McGill University entered into a Non-Disclosure Agreement with Nitragin Holding, Inc., now, by change of name, EMD.

22. Paragraph 2 of the Non-Disclosure Agreement states that, "From and after the date hereof, McGill shall not disclose Confidential Information to any Person, except to its Representatives who need to know such Confidential Information in connection with its investment in AGBH and the sale of the Purchased Shares." Under this Agreement, Confidential Information incorporates Intellectual Property Rights defined in a Main Share Purchase Agreement as information "relating to the ...competitive interests" of EMD.

23. On November 13, 2009, EMD, through its counsel, requested "a full disclosure of all Becker Underwood product offerings that incorporate technology licensed to it by McGill," including the '151 Patent.

24. On November 30, 2009, Becker Underwood responded through its counsel to EMD's November 13, 2009 letter. In that response, Becker Underwood did not provide any of the information or products requested by EMD.

25. Upon information and belief, McGill University has granted to Becker Underwood an exclusive license under the '151 Patent.

COUNT 1

(Patent Infringement)

26. The allegations of paragraphs 1 through 25 are incorporated by reference as though fully set forth herein.

27. Upon information and belief, Becker Underwood, without authority, makes, uses, offers to sell, sells within the United States, and/or imports into the United States, products that incorporate or make use of one or more invention covered by the '018 Patent, including but not limited to its VAULT® HP product, thereby infringing, contributing to the infringement of, and/or actively inducing infringement of one or more claims of the '018 Patent.

28. Upon information and belief, Becker Underwood, without authority, makes, uses, offers to sell, sells within the United States, and/or imports into the United States, products that incorporate or make use of one or more invention covered by the '718 Patent, including but not limited to its VAULT® HP product, thereby infringing, contributing to the infringement of, and/or actively inducing infringement of one or more claims of the '718 Patent.

29. Upon information and belief, Becker Underwood, without authority, makes, uses, offers to sell, sells within the United States, and/or imports into the United

States, products that incorporate or make use of one or more invention covered by the '664 Patent, including but not limited to its VAULT® HP product, thereby infringing, contributing to the infringement of, and/or actively inducing infringement of one or more claims of the '664 Patent.

30. Upon information and belief, Becker Underwood, without authority, makes, uses, offers to sell, sells within the United States, and/or imports into the United States, products that incorporate or make use of one or more invention covered by the '149 Patent, including but not limited to its VAULT® HP product, thereby infringing, contributing to the infringement of, and/or actively inducing infringement of one or more claims of the '149 Patent.

31. Upon information and belief, Becker Underwood, without authority, makes, uses, offers to sell, or sells within the United States, and/or imported into the United States, products that incorporate or make use of one or more invention covered by the '011 Patent, including but not limited to its VAULT® HP product, thereby infringing, contributing to the infringement of, and/or actively inducing infringement of one or more claims of the '011 Patent.

32. Upon information and belief, Becker Underwood had actual knowledge of the '018 Patent, the '718 Patent, the '664 Patent, the '149 Patent, and the '011 Patent. Becker Underwood has not provided any objectively reasonable basis for believing that its VAULT® HP product does not infringe at least one valid U.S. Patent, despite EMD's request. Accordingly, Becker Underwood's infringement of one or more of the above listed patents is intentional and willful.

33. EMD is entitled to damages as a result of the intentional and willful infringement by Becker Underwood.

34. Becker Underwood has caused irreparable damage to EMD by its acts of infringement and will continue its acts of infringement unless permanently enjoined by this Court.

REQUEST FOR RELIEF

WHEREFORE, Plaintiffs EMD respectfully request judgment against Defendant Becker Underwood as follows:

(a) That Becker Underwood has committed acts of patent infringement in violation of the Patent Act, 35 U.S.C. § 271 and that such infringement was willful;

(b) That Becker Underwood, its officers, agents, servants, employees and attorneys, and any other persons in active concert or participation with them, are enjoined from continuing the acts herein complained of, and that Becker Underwood and such other persons be permanently enjoined and restrained from further infringing activities;

(c) Awarding EMD all relief available under the patent laws of the United States, including but not limited to monetary damages, including prejudgment interest and enhanced damages, based on the Defendant's infringement;

(d) Trebling all damages awarded to EMD for Becker Underwood's willful infringement pursuant to 35 U.S.C. § 284;

(e) Finding this to be an exceptional case, and award Plaintiffs its costs and reasonable attorneys' fees in respect thereto in accordance with 35 U.S.C. § 285; and

(f) Granting Plaintiffs such other relief as this Court deems just and equitable.

DEMAND FOR JURY TRIAL

Pursuant to Fed. R. Civ. P. 38(b), Plaintiffs demand a trial by jury on all issues so triable.

Date: January 29, 2010.

Respectfully submitted,

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EXHIBIT A

US005646018A

United States Patent [19]

Broughton et al.

[11] Patent Number: 5,646,018

[45] Date of Patent: Jul. 8, 1997

[54] **BROAD HOST SPECTRUM RHIZOBIACEAE NODULATION SIGNALS**

[75] Inventors: **William John Broughton**, Geneva, Switzerland; **Jean Louis Claude Denarie**, Castanet-Tolosan; **Fabienne Chantal Marie Maillet**, Pompertuzat, both of France; **Neil Philip John Price**, Athens, Ga.; **Danielle Jean Claudine Prome**; **Jean-Claude Adrien Paul Prome**, both of Pechbusque, France; **Biserka Relic**, Geneva, Switzerland; **Franck Jean Bernard Talmont**, Toulouse, France

[73] Assignees: **Institut National de la Recherche Agronomique - I.N.R.A.**; **Centre National de la Recherche Scientifique - C.N.R.S.**, both of Paris Cedex, France

[21] Appl. No.: **356,319**

[22] PCT Filed: **Jun. 29, 1993**

[86] PCT No.: **PCT/FR93/00653**

§ 371 Date: **Feb. 1, 1995**

§ 102(e) Date: **Feb. 1, 1995**

[87] PCT Pub. No.: **WO94/00466**

PCT Pub. Date: **Jan. 6, 1994**

[30] **Foreign Application Priority Data**

Jun. 29, 1992 [FR] France 92 07958

[51] **Int. Cl.**[6] **C07H 3/06**; C12P 19/26; A01N 43/16; A61K 31/715

[52] **U.S. Cl.** **435/84**; 504/117; 504/189; 514/54; 536/123.1

[58] **Field of Search** 435/84, 100, 101; 536/123.1, 127, 128, 55.1, 55.2; 514/54; 504/117, 189

[56] **References Cited**

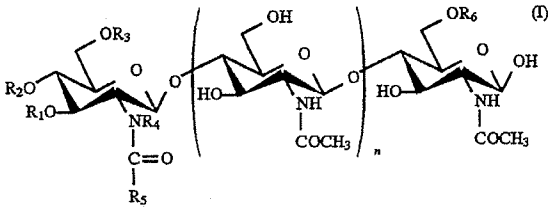
PUBLICATIONS

Bassam et al. (1988) Mol. Plant–Microbe Interactions 1:161–168 Apr. 1988.

Primary Examiner—David Guzo
Assistant Examiner—Robert Schwartzman
Attorney, Agent, or Firm—Oblon, Spivak, McClelland, Maier & Neustadt, P.C.

[57] **ABSTRACT**

Nod factors of general formula (I):



(I)

wherein R_1, R_2 and R_3 are hydrogen atom, a carbamyl group or an acetyl group; R_5 is the aliphatic chain of a fatty acid; n is 1–4; and one or more of substituents R_1, R_2 and R_3 is a carbamyl group, and/or R_4 is a methyl group, and/or R_6 is an optionally substituted monosaccharide or oligosaccharide attached to the glucosamin via a glycoside bond.

17 Claims, 1 Drawing Sheet

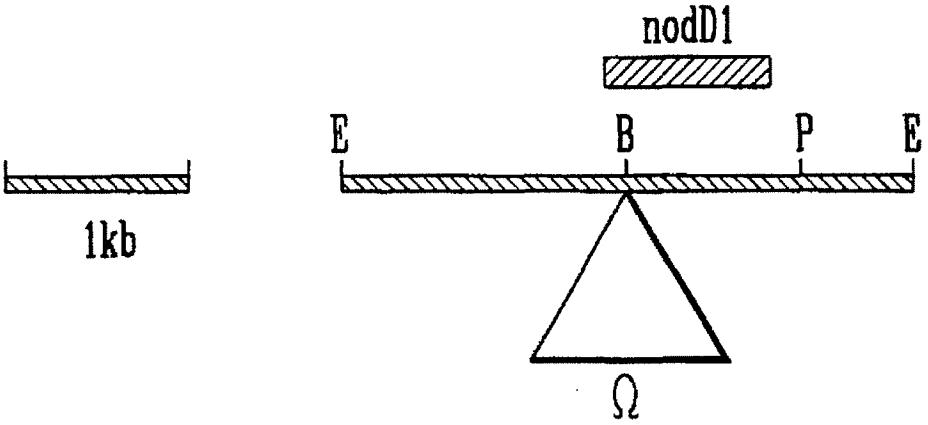


FIG. 1

BROAD HOST SPECTRUM RHIZOBIACEAE NODULATION SIGNALS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to the preparation of novel, broad host spectrum nodulation signals (Nod factors).

2. Description of the Related Art

Sol bacteria which belong to the genera Azorhizobium, Bradyrhizobium, Sinorhizobium and Rhizobium, (which are referred to under the general term rhizobia) are capable of interacting with the roots of legumes in order to form nodules in which they fix atmospheric nitrogen. However, only certain combinations of bacteria and plants result in nodulation and host specificity of rhizobia varies greatly [LONG, Cell. 56, 203 (1989)]; [MARTINEZ et al., Crit. Rev. Plant Sci., 23, 483 (1990)]; [DENARIE et al., in Molecular Signals in Plant-Microbe Communications, D.P.S. Verma Ed. pp. 295–324 (CRC Press, Boca Raton, 1992)]. Certain rhizobia (for example *R. leguminosarum* and *R. meliloti*) form nodules on only a small number of legume species, while, on the other hand, others have a broader host spectrum and can form an association with a large number of plants.

Nodule formation results from a coordinated expression of plant genes and bacterial genes. The expression of rhizobial nodulation genes (nod) is controlled by nodD regulator genes whose products are activated by flavonoids which are secreted by the roots of the plants. The ability of the NodD proteins to interact with the plant flavonoids in a specific manner defines a first level of host specificity.

Moreover, two categories of structural nod genes exist: genes which are in common and specific genes. The nodABC genes are common to all rhizobia, while nod genes, which are specific to the species, are the major determinants of host specificity.

It has been shown that the common nod genes and the specific nod genes are simultaneously involved in the production of extracellular Nod factors which cause deformation of root hairs in legumes. Some inventors have identified Nod factors, termed NodRm, in *R. meliloti* which factors have a lipo-oligosaccharide structure, whose biosynthesis is under the control of common nodABC genes, and which are glucosamine oligomers linked to each other by β-1,4 bonds, N-acylated on the non-reducing terminal glucosamine and N-acetylated on the other glucosamine residues (Application PCT FR/9100283 in the names of the INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE and the CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE). Host specificity is subsequently determined by the nature of the substituents attached to this skeleton which they have in common. In the case of *R. meliloti*, the function of major host specificity genes (nodH and nodPQ) is to determine the sulfation of these lipo-oligosaccharide factors [ROCHE et al., Cell, 67, 1131 (1991)], while, in the case of *R. leguminosarum*, the nodFE genes control the synthesis of a highly unsaturated lipid residue [SPAINK et al., Nature, 354, 125, (1991)].

The strain Rhizobium sp. NGR234 has a unique place amongst the legume symbionts; it has, in fact, the broadest host spectrum of all known rhizobia, and it is known at present that it causes nodulation of over 60 legume species. Amongst these hosts there are, in particular, most of the commercially important legumes such as, for example, soya bean or groundnut. Rhizobium NGR234 can, moreover, cause nodulation of plants which do not belong to the legumes, such as, for example, Parasponia andersonii.

SUMMARY OF THE INVENTION

The inventors have sought to isolate and identify the Nod factors which are responsible for the broad host spectrum of Rhizohium sp. NGR234 and were able to characterize a novel family of Nod factors termed NodNGR factors. These NodNGR factors are lipooligosaccharides which belong to the same family as the NodRm factors which have already been described by some of the inventors (Application PCT FR/9100283), but also have structural characteristics which allow them to be distinguished from Nod Rm factors.

Firstly, their reducing terminal glucosamine residue is substituted on the C6 by a different sugar;

Secondly, their non-reducing terminal glucosamine can be esterified by one or more carbamoyl groups;

Thirdly, the nitrogen atom which is substituted by the long-chain fatty acid is also methylated.

Moreover, the inventors have studied the structure of the Nod factors produced by a range of strains of the Rhizobiaceae from very different geographical origins and which are symbionts of a very wide range of hosts, such as *Rhizobium tropici* which forms nodules on beans and Leucaena, *Sinorhizobium fredii*, which forms nodules on soya beans and *Azorhizobium caulinodans* which is a symbiont of Sesbania. They found that the Nod factors produced by these different strains had at least one of the structural features observed for the NodNGR factors such as the presence of a sugar on the reducing terminal glucosamine (*Sinorhizobium fredii, Azorhizobium caulinodans, Rhizobium phaseoli*) or of an N-methyl group on the non-reducing terminal glucosamine (*Rhizobium tropici, Azorhizobium caulinodans*).

The novel Nod factors of the invention, which show at least one of the three structural characteristics mentioned hereinabove, will generally be termed hereinafter NodNGR-type Nod factors.

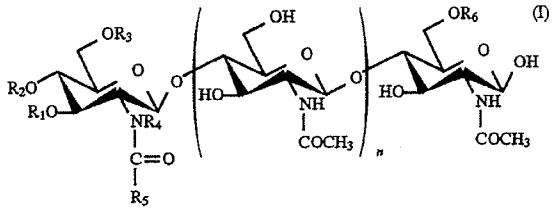
BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts the restriction map of the Eco RI fragment containing the nodD1 region from NGR234.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

More particularly, the NodNGR-type Nod factors obtained from NGR234 will hereinbelow be termed NodNGR factors. Nod factors obtained from other strains studied by the inventors and having at least one of these characteristics represent NodNGR-type Nod factors. It seems that the source of NodNGR-type Nod factors can be very varied.

The present invention relates to Nod factors of the general formula (I) hereinbelow:



(I)

in which:

R_1, R_2 and R_3 represent a hydrogen atom, a carbamoyl group or an acetyl group;

R_5 represents the aliphatic chain of a fatty acid;

n is between 1 and 4, and wherein:

one or more of the substituents R_1, R_2 or R_3 is a carbamoyl group, and/or

R_4 represents a methyl group, and/or

R_6 represents a monosaccharide or an oligosaccharide, optionally substituted, and linked to the glucosamine by a glycosidic linkage.

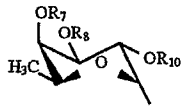
In a preferred embodiment of the present invention , R_5 represents the aliphatic chain of a C_{10-24}-, preferably a C_{14-20}-, fatty acid.

In a preferred form of this embodiment, R_5 is the aliphatic chain of vaccenic acid or palmitic acid.

Vaccenic acid and palmitic acid are major substituents found in preparations of NodNGR factors from Rhizobium NGR234; however, a large number of different fatty acids varying as much with regard to the number of carbon atoms of the aliphatic chain as by the degree of unsaturation, or by the presence of substituents such as hydroxyl groups on said aliphatic chain, was observed; no effect of these variations on the activity of the NodNGR factors was found by the inventors.

In yet another preferred embodiment of the present invention, R_6 is selected from the monosaccharide group comprising optionally substituted fucose and optionally substituted arabinose.

In an advantageous arrangement of this embodiment, R_6 has the general formula (II):



(II)

in which:

R_7 and R_8 represent a hydrogen atom, an acetyl group or a sulfate group,

R_{10} represents a hydrogen atom or a methyl group.

In a preferred form of this arrangement, R_7 is a hydrogen atom and R_8 a sulfate group.

In another preferred form of this arrangement, R_7 is an acetyl group and R_8 a hydrogen atom.

In yet another preferred form of this arrangement, both R_7 and R_8 are hydrogen atoms.

The NodNGR factors belong to the same family of molecules as the NodRm factors which have been purified from *R. meliloti*: all have in common the skeleton of D-glucosamine residues linked to each other by β-1,4 linkages, are N-acylated on the non-reducing terminal glucosamine and N-acetylated on the other residues. These structural similarities tie in with the observations made before by some of the inventors and confirm that, in all rhizobia, the function of the nodABC genes is to control the synthesis of a skeletal structure of N-acylated and N-acetylated oligochitosan. The work carried out by the inventors therefore reveals that the Nod factors of all Rhizobiaceae belong to the same chemical family.

In the NodRm factors, the fatty acid chain contains at least one conjugated double bond which seems to play an essential role in the induction of nodular meristems; in contrast, the NodNGR factors are N-acylated with vaccenic acid or palmitic acid and therefore do not have a conjugated double bond. The nitrogen atom which is substituted by a long-chain fatty acid is also methylated in the NodNGR factors. These observations allow the hypothesis to be put forward that certain biological activities of the Nod-type lipo-oligosaccharides require a certain structural configuration at the joint between the lipid part and the saccharide skeletal structure. These structural conditions would be provided in one case by the conjugated double bond and, in the other case by the N-methyl group.

The family of the NodNGR factors is very large. Mass spectrometry analysis using fast-atom bombardment ionization (FAB-MS) and also nuclear magnetic resonance analysis have shown that the following variations exist in the substituents:

1) The 2-O-methylfucose residue can be unsubstituted or else sulfated on O-3 or acetylated on O-4;
2) The nitrogen atom of the non-reducing terminal glucosamine can be acylated by palmitic acid or else by vaccenic acid;
3) The number of carbamoyl substituents on the non-reducing terminal glucosamine varies between zero and two.

The combinations of these possible different substituents lead to at least 18 (=3×2×3) possible structures if the carbamoyl substitution sites are fixed and their number may even be greater to the extent that the carbamoyl group substitution site, or sites, can vary between positions O-3, O-4 and O-6. It is reasonable to assume that it is in particular this structural diversity which is responsible for the broad host spectrum of the NodNGR factors.

The invention also relates to rhizobia strains which overproduce NodNGR type factors, which comprise at least one recombinant plasmid expressing a regulator gene nodD from NGR234 and in particular a rhizobium strain NGR234 which overproduces Nod factors, which strain is obtained by introducing, into NGR234, a recombinant multicopy plasmid termed pA28 which expresses a regulator gene nodD from NGR234, so as to increase the number of copies of this gene, which results in an at least 10-fold increase of the amount of Nod factors produced.

The invention also encompasses recombinant plasmids carrying the regulator gene nodD1 from NGR234, in particular plasmid pA28, which results from inserting an EcoRI-PstI fragment from plasmid pNGRH6 [BASSAM et al. Mol. Plant-Microbe Interact, 1, 161, (1988)], which carries the nodD1 region from NGR234, into plasmid pRK7813 [JONES and GUTTERSON, Gene, 61, 299–306, (1987)].

The invention also relates to a process for the preparation of NodNGR factors, or NodNGR-type factors, which process comprises a step in which at least one strain of rhizobia producing said Nod factors is cultured.

Preferably, a strain will be chosen into which a plasmid according to the invention has been introduced.

In a preferred embodiment of the preparation process of NodNGR-type factors according to the invention, it comprises, moreover, a step in which one or more fractions comprising said factors are extracted from said culture of rhizobia strains.

In a preferred arrangement of this embodiment, the NodNGR factors are extracted from culture supernatant by reverse-phase chromatography, by absorption on a silica column to which hydrophobic groups, such as octadecyl residues, are grafted, followed by elution with methanol.

The present invention also relates to a plant treatment agent comprising, as active ingredient, at least one NodNGR factor or NodNGR-type factor as defined further above, which can be used in particular:

as an agent for stimulating symbiotic properties of legumes, especially with regard to nitrogen fixation;

as an agent for stimulating the defence mechanisms of plants against pathogene.

Said plant treatment agent preferably comprises a mixture of NodNGR factors and/or NodNGR-type factors. It can advantageously also comprise other Nod factors, for example NodRm-type factors.

In an advantageous embodiment of the plant treatment agent according to the present invention, the composition is included in a solid carrier, such as granules, or else formulated in the form of a coating composition for seed or an aqueous solution or suspension for spraying, in which a Nod factor or Nod factors, according to the invention are present alone or in association with other components, such as, for example, other Nod factors.

In another advantageous embodiment of the plant treatment agent according to the present invention, a Nod factor, or each of the ingredients of a mixture of Nod factors, according to the invention are present in the coating compositions or in the aqueous solutions or suspensions at a concentration of between 10^{-6}M and 10^{-14}M.

Moreover, the present invention relates to a therapeutic agent comprising, as active ingredient, at least one NodNGR factor or NodNGR-type factor as defined further above.

In an advantageous embodiment of this therapeutic agent, said factor is present in the therapeutic agent at a concentration of between 10^{-4}M and 10^{-8}M.

Besides the above arrangements, the invention also encompasses other arrangements which will emerge from the description which follows.

It must be understood, however, that these examples as well as the appended drawings are given only by way of illustrating the subject of the invention but without imposing any limitation whatsoever.

EXAMPLE 1

PRODUCTION OF A STRAIN OF RHIZOBIACEAE BACTERIA WHICH OVERPRODUCE NodNGR FACTORS

Strain NGR234 and its DNA were engineered as described by BROUGHTON et al. (Arch. Microbial. 141, 14 (1985)) and PERRET et al. (Proc. Natl. Acad. Sci. 88, 1923 (1991)), or by means of traditional techniques (J. SAMBROOK et al., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989)).

A 2.9 kb EcoR1 fragment containing the nodD1 region from NRG234 was excised from plasmid pNGRH6 [BASSAM et al., Mol., Plant-Microbe Interact. 1, 161, (1988)] and then digested with Pst1. The resulting 2.2 kb EcoR1-Pst1 fragment was cloned into pRK7813 at the Pst1 site to give plasmid pA28. pA28 is reintroduced into Rhizobium sp. NGR234 (Rif^R) by means of conjugation. The resulting strain NGR(pA28) overproduces Nod factors. It is Nod^+ on *L. leucocephala, M. atropurpureum,* and *V. unguiculata*. FIG. 1 shows the restriction map of the EcoR1 fragment. The restriction sites are designated as follows:

B = BamH1,	C = Cla1,	E = EcoR1,
P = Psi1,	S = Sst1.	

EXAMPLE 2

PURIFICATION OF THE NodNGR FACTORS

The bacteria NGR234 or NGR234(pa28) are cultured at 27° C. on B+D medium (W. J. BROUGHTON and M. J. DILWORTH, Biochem. J. 125, 1075 (1971)) containing 12 mM succinate, 6 mM glutamate, 1 ml/litre of GAMBORG's Vitamin B5 solution (Sigma, St. Louis, Mo.) (=RMM3) to the end of the logarithmic phase.

For analysis by thin-layer chromatography, 30 ml of cultured bacteria (NGR 234) are induced using 10^{-6}M of apigenin, which induces the expression of the nodD genes from NGR234, in the presence of 1 μCi/ml of sodium sulfate labeled with sulfur-35, or sodium acetate labeled with carbon-14.

The supernatants are extracted on SEPPAK C_{18} cartridges (Waters Assoc., Milford, Mass.), washed with distilled water and eluted using methanol. The concentrated methanol extracts are applied to silica gel 60 G plates (MERCK, DARMSTADT); chromatography is carried out in a chloroform: methanol: 5M NH_4OH (5:4:1 by volume) mixture, and the plate is placed on a FUJI RX film (Fuji Photo Film Co, TOKYO).

Induction of nod genes by 10^{-6}M apigenin in the presence of ^{14}C-labeled acetate or ^{35}S-labeled sulfate results in two radioactive spots on the TLC plates. Extraction of the active principles contained in each of these spots allows substances to be obtained which cause deformation of the root hairs of Macroptilium. This demonstrates that NGR234 secretes sulfur-containing Nod factors. On the other hand, no spot obtained from an NGR234 derivative can be detected in which the nodABC genes have been deleted. Moreover, if the number of copies of the nodD1 gene (regulator gene) is increased by introducing plasmid pA28 into NGR234, an increased production of Nod factors is observed, the Mount produced being multiplied by a factor of 5 to 10.

For chemical analysis, the Nod factors were isolated from the culture supernatant of NGR234 containing plasmid pA28, following induction with apigenin. This production on a larger scale is effected using 50 litres of NGR234(pa28) bacterial culture which has previously been induced with apigenin in RMM3 medium. The lipophilic material is recovered on a C_{18} reversed-phase column (LICHROSORB-18, 40 μ, MERCK, DARMSTADT). The column is washed with 50 times its volume of distilled water and eluted with 10 volumes of methanol. The methanolic solution is evaporated in vacuo and diluted with 100 ml of distilled water. After filtration, the aqueous solution is extracted using 50 ml of ethyl acetate to extract, in particular, apigenin. The aqueous solution is concentrated, and the water-soluble constituents are separated by preparative HPLC on a C_{18} reversed-phase column. Elution is monitored at 206 nm. The solvent is a gradient of acetonitrile in water.

Two major peaks termed fractions A and B were collected in this way. Fraction A (0.3 mg/l of the starting culture) co-elutes with the material originating from the culture labeled with sulfur-35, while fraction B (0.5 mg/l of the original culture) is not labeled under these conditions. The comparison of biological activities of these two fractions is described hereinbelow in Example 4.

EXAMPLE 3

CHARACTERIZATION OF THE NodNGR FACTORS

Hydrolysis with trifluoroacetic acid (4M for 4 hours at 100° C.) of each of the two fractions liberates sugars and fatty acids. The sugars were identified as D-glucosamine, N-methyl-D-glucosamine and 2-O-methyl-L-fucose, either by gas chromatography, mass spectrometry, (GC-MS) of their alditol acetate derivatives, or else by gas chromatographic analysis of their (+)-2-butanolglycosides. Two acids were identified: the largest component as being vaccenic acid (11-Z-octadecenoic acid), while the minor component

(approximately 20% of the total) was identified as palmitic acid. The existence of a skeleton which they have in common and which is composed of pentameric N-acetylglucosamine oligomers having a plurality of substituents was deduced from the FAB mass spectrum, which reveals series of ions separated by 203 mass units (molar mass of an N-acetylglucosamine residue).

Fraction A is a mixture of a plurality of sulfated compounds, as confirmed by the ease with which the SO_3 radical is lost in positive ionized form. The molecular weight of the major component, deduced from the spectrum of negative ions, is 1595. Other components with a mass of less than 43 or 26 mass units, or a combination of the two, were detected. This latter difference corresponds to the difference between the molecular weight of vaccenic acid and the molecular weight of palmitic acid. Equally, the difference of 43 mass units, which was repeated twice, was attributed to the presence or absence of additional CO—NH groups (carbamoyl residues). The fact that this pattern of three peaks which are separated by 43 mass units accompanied by satellite peaks at a distance of 26 mass units is observed each time a glycosidic linkage is ruptured in the form of positive ions (formation of oxenium ions) justifies the localization of carbamoyl groups of the non-reducing terminal glucosamine. Moreover, if the mass of the oxenium ions of m/z 440, 483 and 526 is subtracted from the mass of a vaccenyl residue (ketene) and, if appropriate, the mass of zero, one or two carbamoyl groups (43 mass units), the mass of the oxenium ion of a methylglucosamine is obtained. This allows the N-methylglucosamine to be localized at the non-reducing end of the oligosaccharide.

Fraction B is also a mixture. Two major components were identified (molecular weights 1557 and 1515, respectively). The difference of 42 mass units between these two components suggests that the second is a monoacetylated form of the first. On the other hand, as in fraction A, other components in which the mass is lower than 43 or 26 mass units are also present. As in the case of the components of fraction A, the carbamoyl groups are localized on the non-reducing terminal N-methylglucosamine which has carries the N-acyl group. In contrast, the additional acetyl group, which is not present in any of the oxenium ions observed, is localized near the reducing end.

The carbon-13 NMR spectrum is compatible with the presence of carbamoyl groups (δ=161.09, 160.62 and 159.80 ppm) and the presence of the other substituents described above. The proton NMR spectrum attributes β configurations to the linkages between glucosamines and α configurations for the linkage between 2-O-methylfucose and the reducing glucosamine. The COSY spectrum shows a correlation between the H-5 of the fucose and a deshielded proton δ=4.53 ppm in the case of the compounds of fraction B. This allows the position of the acetyl group to be attributed to the 0-4 position of 2-O-methylfucose. In parallel, the COSY spectrum of the compound of fraction A shows a correlation between the H-2 of 2-O-methylfucose (3.67 ppm) and the deshielded H-3 proton at δ=4.65 ppm, which allows the sulfate group to be localized at the O-3 of 2-O-methylfucose. This latter attribution is confirmed by analysis of the sugars obtained by hydrolysis of the reduced and permethylated fraction A, which shows the presence of dimethyl-2,4-fucose. Moreover, by identifying trimethyl-1,2,3,5-glucosaminitol in the hydrolysis products of the reduced and permethylated fractions A and B, it can be confirmed that the methylfucose is linked glycosidically to the O-6 of the reducing glucosamine. Finally, this analysis also shows 1–4 linkages between the various glucosamines. Since the methylation conditions result in the simultaneous elimination the ester groups (acetates and carbamates), the position of these groups cannot be determined by this method. However, the major components of fractions A and B are bicarbamylated while the types which lack carbamoyl substituents are in the minority.

EXAMPLE 4

TEST FOR GROWTH OF ROOT HAIRS (Hai) AND DEFORMATION OF ROOT HAIRS (Had) CAUSED BY NOD-SULFATED AND NON-SULFATED FACTORS FROM RHIZOBIUM NGR234 ON MACROPTILIUM ATROPURPUREUM, MEDICAGO SATIVA, VICIA SATIVA AND VIGNA UNGUICULATA

The two fractions A and B, (sulfated and non-sulfated) were separated by a reverse-phase HPLC chromatography following the protocol described in Example 2 and were tested separately for their biological activity.

The Had test (deformation of root hairs) on *M. sativa* and Hai test (proliferation and bending of the root hairs) on *V. sativa* were carried out as described by ROCHE et al. [Cell., 76, 1131 (1991)]. In the Bad tests on Macroptilium and Vigna, sterile plantlets are placed into modified Eppendorf tubes (with the cap and part of the bottom removed), and the Eppendorf tubes containing the plantlets are suspended into test tubes whose bottom is painted black in order to protect the roots from light, in such a manner that the root tip is in contact with 10 ml of B+D medium. After incubation for 60 hours (16-hour day, 30° C.; 8-hour night, 20° C.), the roots are removed, stained with Methylene Blue and examined under an inverted microscope. Those root systems which clearly show branching or bending (prolific ramifications or bending at more than one point in the root system) are termed Had^+. Those roots which are covered in root hairs are termed Hai^+. 10 plants were used for each treatment and dilution. Moreover, 40 (Macroptilium and Vigna) and 60 (Medicago and Vicia) plantlets are used as control plants (grown on medium only) in order to estimate the intrinsic variability of the characters Bad and Hai between one plant and another.

The results are shown in Table I hereinbelow. These results show the number of plants (above 10) which show a positive Bad or Hai activity. The numbers are followed by ^{S} if the ratio of Had^+ or Hai^+ is significantly higher (probability P=0.05) in the treated plants than in the controls (analyzed using the Fisher test).

NodRm-IV (Ac,S) is the major sulfated Nod factor of strain *R. meliloti* 2011. It is used as positive control for Bad activity on Medicago.

NodRm-IV(Ac) is a non-sulfated Nod factor of $NodH^-$ mutants of *R. meliloti*. It is used as positive control for Hai activity on Vicia.

TABLE 1

	CONCENTRATION Nod FACTOR (M)						
	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}
Macroptilium (Had)							
NodNRG sulfated	nt	10^{5}	10^{a}	7^{a}	3^{a}	0	
NodNGR non-sulfated	nt	10^{5}	9^{a}	4^{a}	1	0	
Vigna (Had)							
NodNRG sulfated	10^{a}	10^{a}	10^{a}	10^{a}	10^{a}	9^{a}	6^{a}
NodNGR non-sulfated	10^{a}	10^{a}	10^{a}	8^{a}	5^{a}	4	nt
Medicago (Had)							
NodNRG sulfated	9^{a}	8^{a}	9^{a}	5^{a}	3^{a}	1	
NodNGR non-sulfated	5^{a}	1	2	0	1	1	
NodRM-IV (AC,S)	nt	9^{a}	8^{a}	7^{a}	6^{a}	4^{a}	
Vicia (Hai)							
NodNRG sulfated	4^{a}	5^{a}	3^{a}	0	0	0	
NodNGR non-sulfated	10^{a}	10^{a}	9^{a}	2^{a}	0	0	
NodRM-IV (Ac)	nt	10^{a}	10^{a}	8^{a}	2^{a}	0	

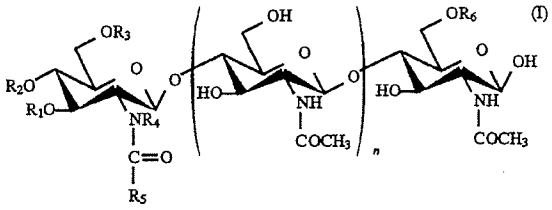
nt = non tested

In root hair deformation tests (Had) carried out with host plants of NGR234, the two groups of NodNGR factors are active at concentrations of as little as 10^{-10}M/10^{-11}M in Macroptilium and 10^{-11}M/10^{-12}M in Vigna. Moreover, in Vigna, the NodRm factors induce not only deformations of the hairiness of the roots, but also of the appearance of a large number of root hairs, as well as bending of the root hairs (Hai).

The sulfated NodRm factors obtained from *R. meliloti* are Had^{+} in *Medicago sativa*, and Hai^{-} in Vicia sativa supsp. nigra. In contrast, the non-sulfated NodRm factors secreted by $NodH^{-}$ mutants of *R. meliloti* are Had^{-} in Medicago and Hai^{+} in Vicia. Interestingly, sulfated and non-sulfated NodNGR factors have a biological activity on both legumes. In Medicago, the sulfated NodNGR factors are 10,000 times more active than the non-sulfated factors and cause deformation of the hairiness of the roots at concentrations of less than 10^{-11} mole. The sulfated NodNGR factors differ from the sulfated NodRm factors with regard to a large number of criteria: for example, the presence of carbamoyl groups and of a methylfucose residue, localization of the sulfate group on the fucose instead of the glucosamine, the absence of a conjugated double bond on the acyl chain which substitutes the nitrogen, and the presence of a methyl group which substitutes the nitrogen. However, both types of factors are active in Medicago, and their activity decreases by a factor of approximately 10,000 when the sulfate group is removed, which demonstrates that Medicago is highly sensitive to sulfated Nod factors. In contrast, in Vicia, the non-sulfated compounds are more active, and deformation of the hairiness of the roots is observed at concentrations of less than 10^{-11}M.

We claim:

1. A NodNGR-type factor of the following formula (I):



(I)

in which:

R_1, R_2 and R_3 represent a hydrogen atom, a carbamoyl group or an acetyl group;

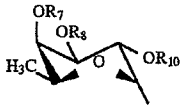
R_5 represents the aliphatic chain of a fatty acid;

n is between 1 and 4, and wherein:

one or more of the substituents R_1, R_2 or R_3 is a carbamoyl group, and/or

R_4 represents a methyl group, and/or

R_6 has the general formula (II)



(II)

in which:

R_7 and R_8 represent a hydrogen atom, an acetyl group or a sulfate group,

R_{10} represents a hydrogen atom or a methyl group.

2. The Nod factor as claimed in claim 1, wherein R_5 represents the aliphatic chain of a C_{10-24} fatty acid.

3. The Nod factor as claimed in claim 1, wherein R_7 is a hydrogen atom and R_8 is a sulfate group.

4. The Nod factor as claimed in claim 1, wherein R_7 is an acetyl group and R_8 is a hydrogen atom.

5. The Nod factor as claimed in claim 1, wherein both R_7 and R_8 are hydrogen atoms.

6. A process for the preparation of NodNGR-type factors as claimed in claim 1, which process comprises a step in which at least one strain of Rhizobiaceae producing said Nod factors in cultured, and a step in which said NodNGR factors are purified from the culture.

7. Plant treatment agent, which comprises, as active ingredient, at least one NodNGR-type factor or a mixture of NodNGR-type factors as claimed in claim 2.

8. The Nod factor as claimed in claim 2, wherein R_5 is the aliphatic chain of vaccenic acid or palmitic acid.

9. The Nod factor as claimed in claim 2, wherein R_5 represents the aliphatic chain of a C_{14-20} fatty acid.

10. The process as claimed in claim 6, wherein said strain is a strain which overproduces Nod NGR factors and comprises at least one plasmid encoding nodD1 from Rhizobium, Azorhizobium, Bradyrhizobium or Sinorhizobium.

11. The plant treatment agent as claimed in claim 7, which comprises, additionally, other Nod factors.

12. The plant treatment agent as claimed in claim 7, which is included in a solid carrier or formulated in the form of a coating composition for seed or an aqueous solution or suspension for spraying.

13. The plant treatment agent as claimed in claim 7, wherein the NODNGR-type factor or each of the ingredients of the mixture of NodNGR-type factors is present at a concentration of between 10^{-6}M and 10^{-14}M.

14. The process as claimed in claim 10, wherein the plasmid results from inserting an Eco I-Pst I fragment of plasmid pNGRH6 comprising the regulator gene nodD1 from strain NGR234 into plasmid pRK7813.

15. A strain of Rhizobiaceae which overproduces NodNGR-type factors for carrying out the process as claimed in claim 10, which comprises at least one recombinant plasmid which results from inserting an EcoRI-PstI fragment of plasmid pNGRH6 comprising a regulator gene nodD1 from strain NGR234 into plasmid pRK7813.

16. The process as claimed in ether of claims 6 or 14, which comprises, moreover, a step in which one or more fractions comprising the NodNGR-type factors are extracted from said culture of Rhizobiaceae.

17. The process as claimed in claim 16, wherein the NodNGR-type factors are extracted from culture supernatant by absorption onto a silica column to which hydrophobic groups are grafted, following by elution with methanol.

* * * * *

EXHIBIT B

US005549718A

United States Patent [19]

Lerouge et al.

[11] Patent Number: 5,549,718

[45] Date of Patent: Aug. 27, 1996

[54] **SUBSTANCE WITH LIPO-OLIGOSACCHARIDE STRUCTURE CAPABLE OF ACTING AS PLANT-SPECIFIC SYMBIOTIC SIGNALS, PROCESSES FOR PRODUCING THEM AND THEIR APPLICATIONS**

[75] Inventors: **Patrice Lerouge**; **Philippe Roche**, both of Toulouse; **Catherine Faucher**; **Fabienne Maillet**, both of Ramonville Saint Agne; **Jean Denarie**, Castanet-Tolosan; **Jean-Claude Promé**, Pechbusque; **Georges Truchet**, Castanet-Tolosan, all of France

[73] Assignees: **Centre National de la Recherche Scientifique (C.N.R.S.)**, Paris; **Institut National de la Recherche Agronomique (I.N.R.A.)**, Paris Cedex, both of France

[21] Appl. No.: **315,491**

[22] Filed: **Sep. 30, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 214,676, Mar. 21, 1994, abandoned, which is a continuation of Ser. No. 930,662, filed as PCT/FR91/00283 on Apr. 5, 1991, abandoned.

[30] **Foreign Application Priority Data**

Apr. 6, 1990 [FR] France 90 04764

[51] **Int. Cl.**^{6} **A01C 1/06**; A01H 3/04; C12P 19/04; C12R 1/41

[52] **U.S. Cl.** **47/57.6**; 47/58; 536/17.2; 536/22.1; 536/123.1; 435/84; 435/172.3; 435/252.2; 435/878; 514/54

[58] **Field of Search** 536/17.2, 22.1, 536/123.1; 435/84, 252.2, 878, 172.3; 514/54; 47/58, 57.6

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Primary Examiner—David T. Fox

Attorney, Agent, or Firm—Oblon, Spivak, McClelland, Maier & Neustadt, P.C.

[57] **ABSTRACT**

An essentially pure substance has a structure of a Nod factor or one of its analogues. The Nod factor is characterized by the fact that its biosynthesis is controlled by at least one nodulation gene (nodA,B,C) common to the Rhizobiaceae, in particular to the genera Rhizobium, Bradyrhizobium, Sinorhizobium and Azorhizobium. This substance consists of a lipo-oligosaccharide which is not a derivative of the exopolysaccharides and which has the general formula (I). In formula (I), the Nod factor of which it has the structure is a plant-specific symbiotic signal and is capable of enhancing the capacity of the bacteria of infect the host plant with which it is associated and/or of accelerating the formation of nodules on the host plant with which it is associated and/or of inducing the transcription of symbiotic genes of the leguminoseae. Applications to the treatment of plants and as an active therapeutic agent in humans and animals. G stands for a hexosamine variously substituted, for example by an acetyl group on the nitrogen, a sulphate group, an acetyl group and/or an ether group on oxygen R_1, R_2, R_3, R_5, R_6, R_7, which may be identical or different, stand for H, CH_3CO—, C_xH_yCO— where X is a whole number between 0 and 17 and Y is a whole number between 1 and 35, or any other acyl group, for example a carbamyl groups, R_4 stands for a saturated or mono-, di or tri-unsaturated aliphatic chain containing at least 12 carbon atoms and n is a whole number between 1 and 4.

11 Claims, 18 Drawing Sheets

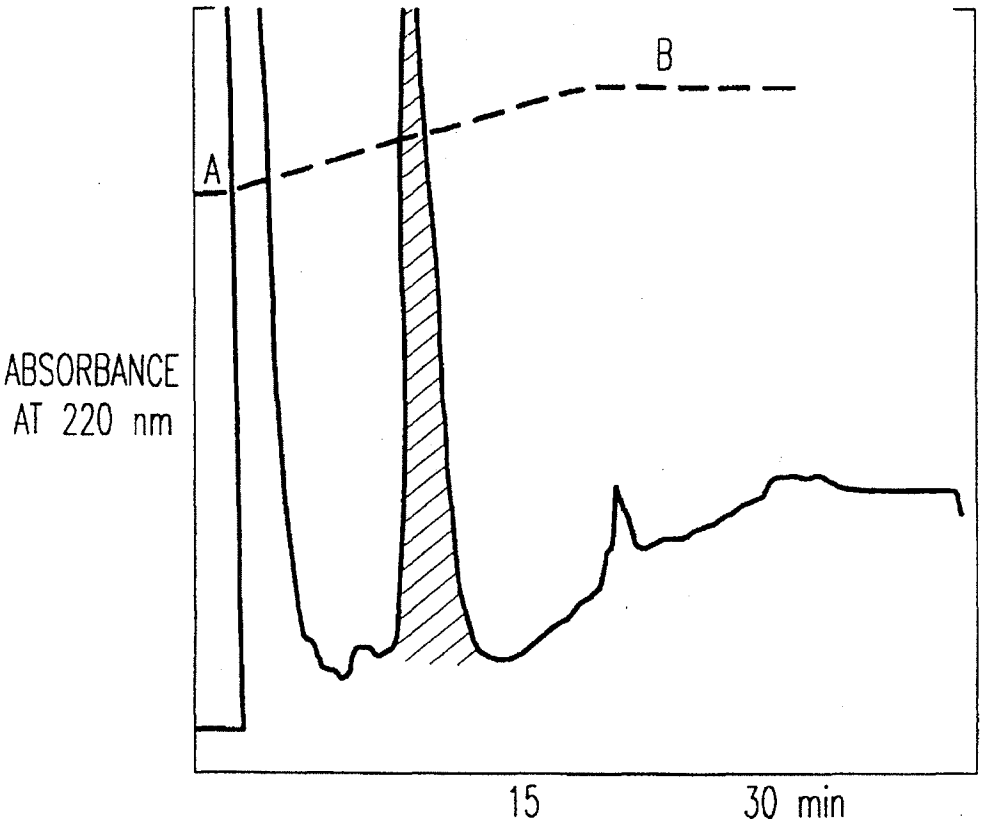


FIG. 1A

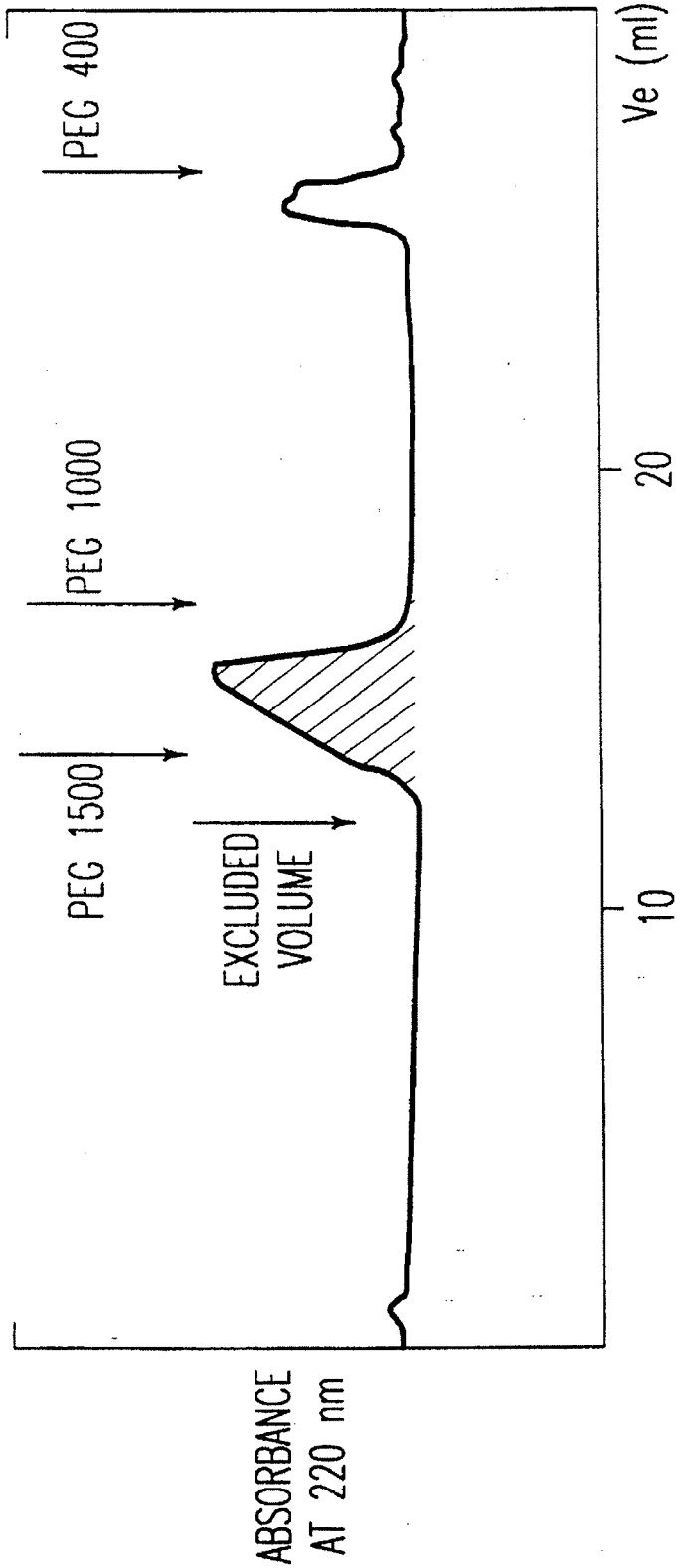

PEG 400
PEG 1000
PEG 1500
EXCLUDED VOLUME
ABSORBANCE AT 220 nm
10
20
Ve (ml)

FIG. 1B

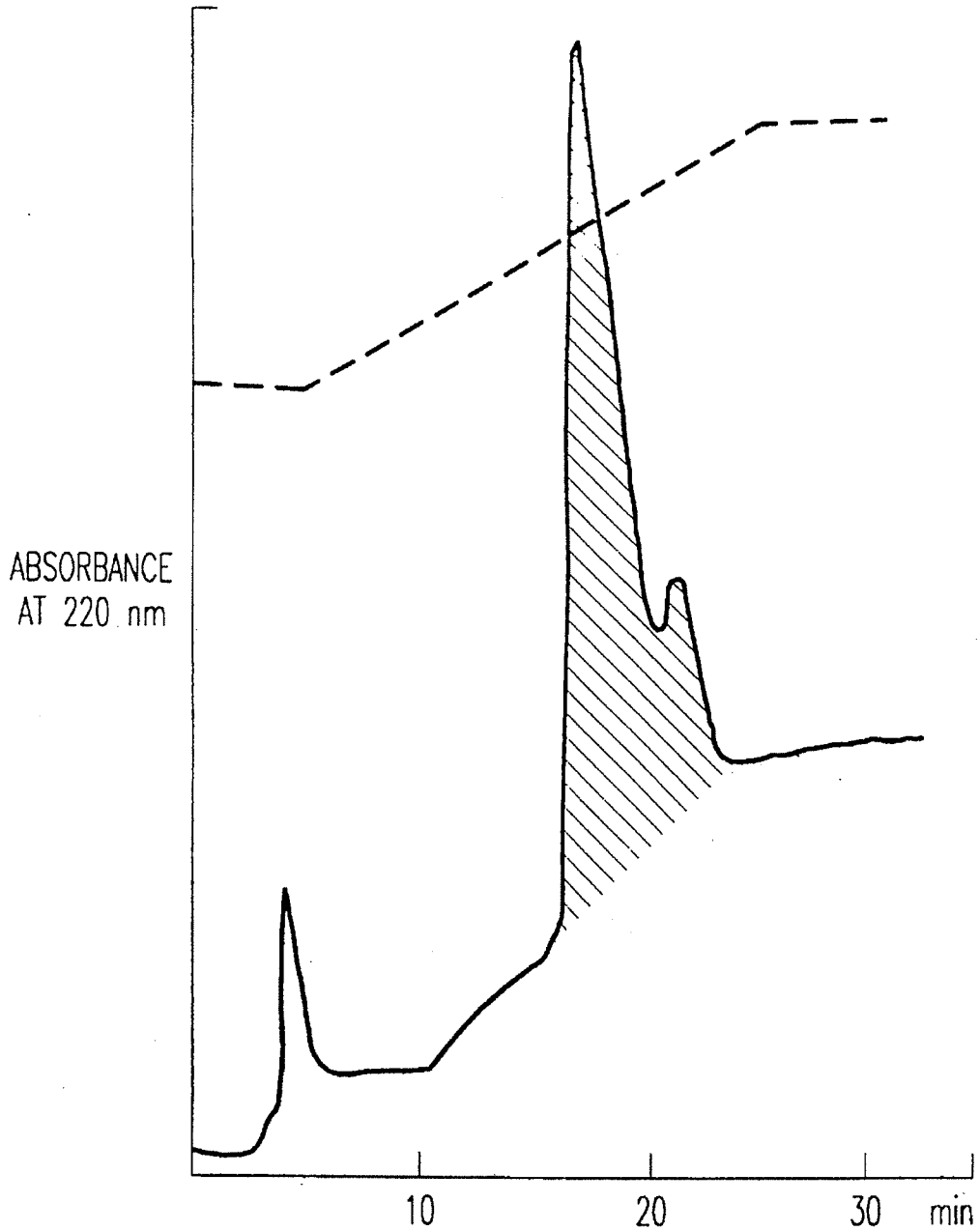


FIG. 1C

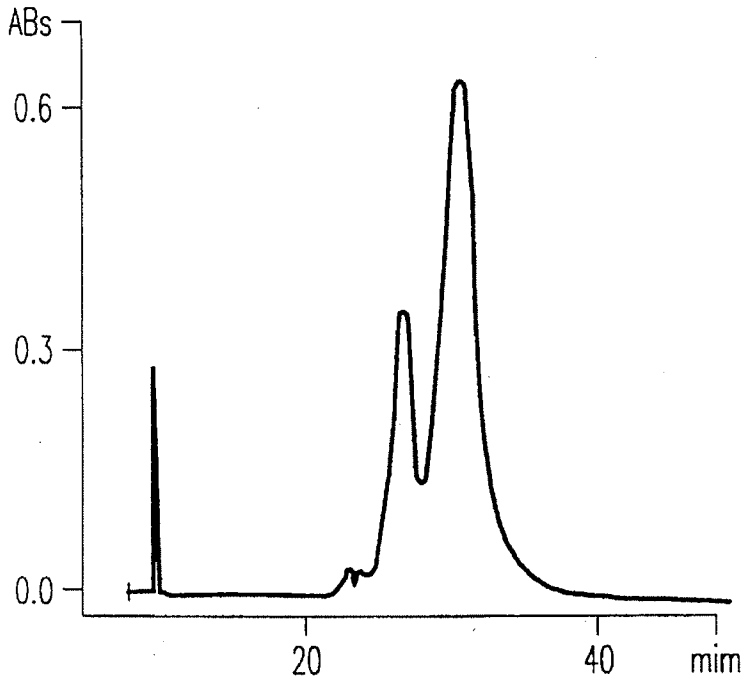


FIG. 1D

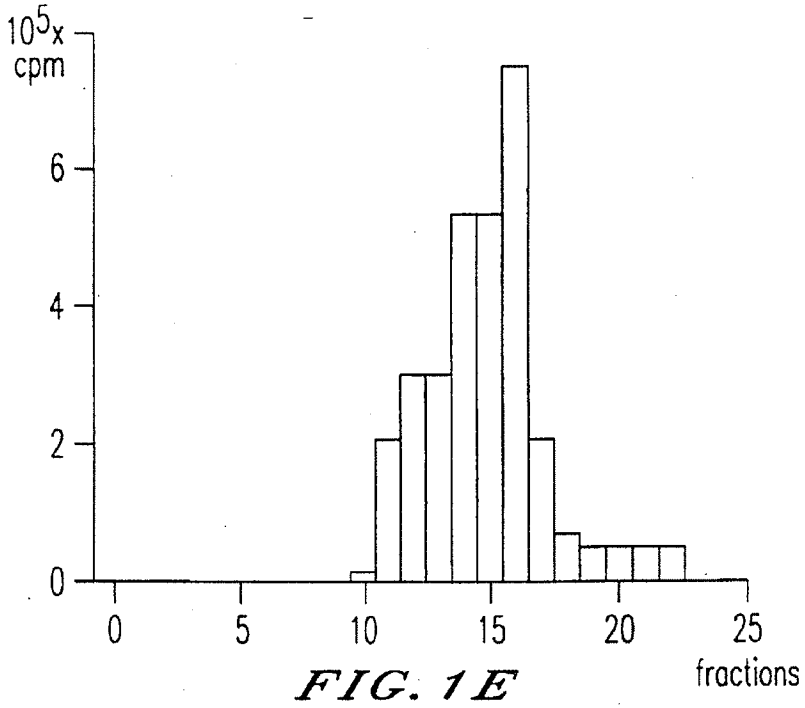


FIG. 1E

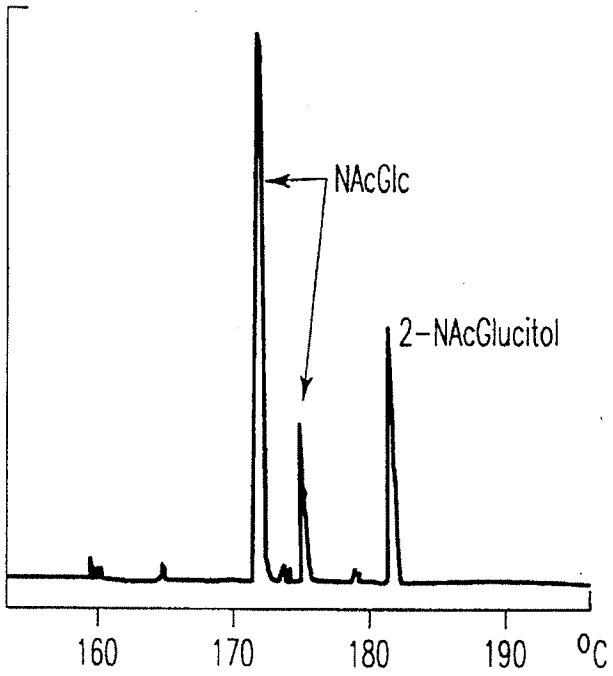

NAcGlc
2-NAcGlucitol
160
170
180
190
°C

FIG. 2A

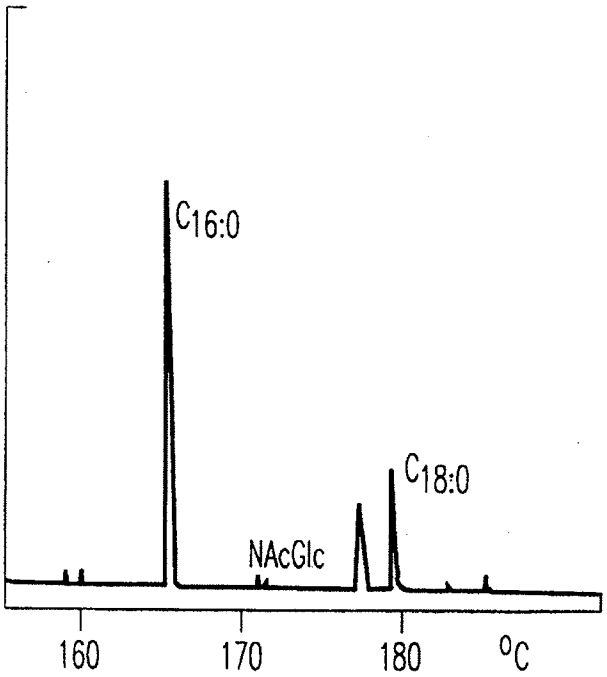
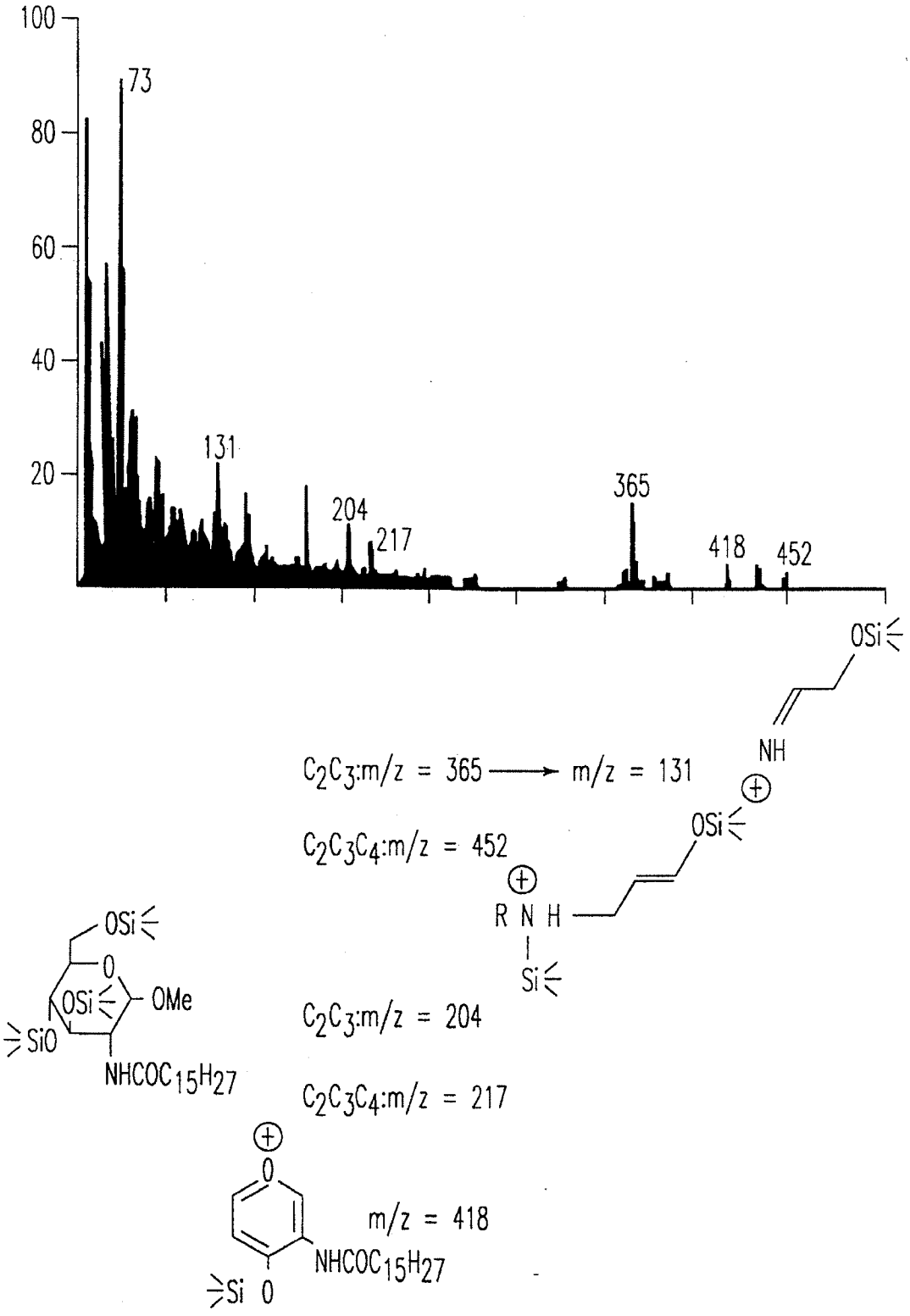

C16:0
C18:0
NAcGlc
160
170
180
°C

FIG. 2B

FIG. 2C


100
80
60
40
20
73
131
204
217
365
418
452
OSi
NH
C_2C_3:m/z = 365 → m/z = 131
$C_2C_3C_4$:m/z = 452
R N H
Si
OSi
O
OSi
OMe
SiO
$NHCOC_{15}H_{27}$
C_2C_3:m/z = 204
$C_2C_3C_4$:m/z = 217
O
m/z = 418
$NHCOC_{15}H_{27}$
Si O

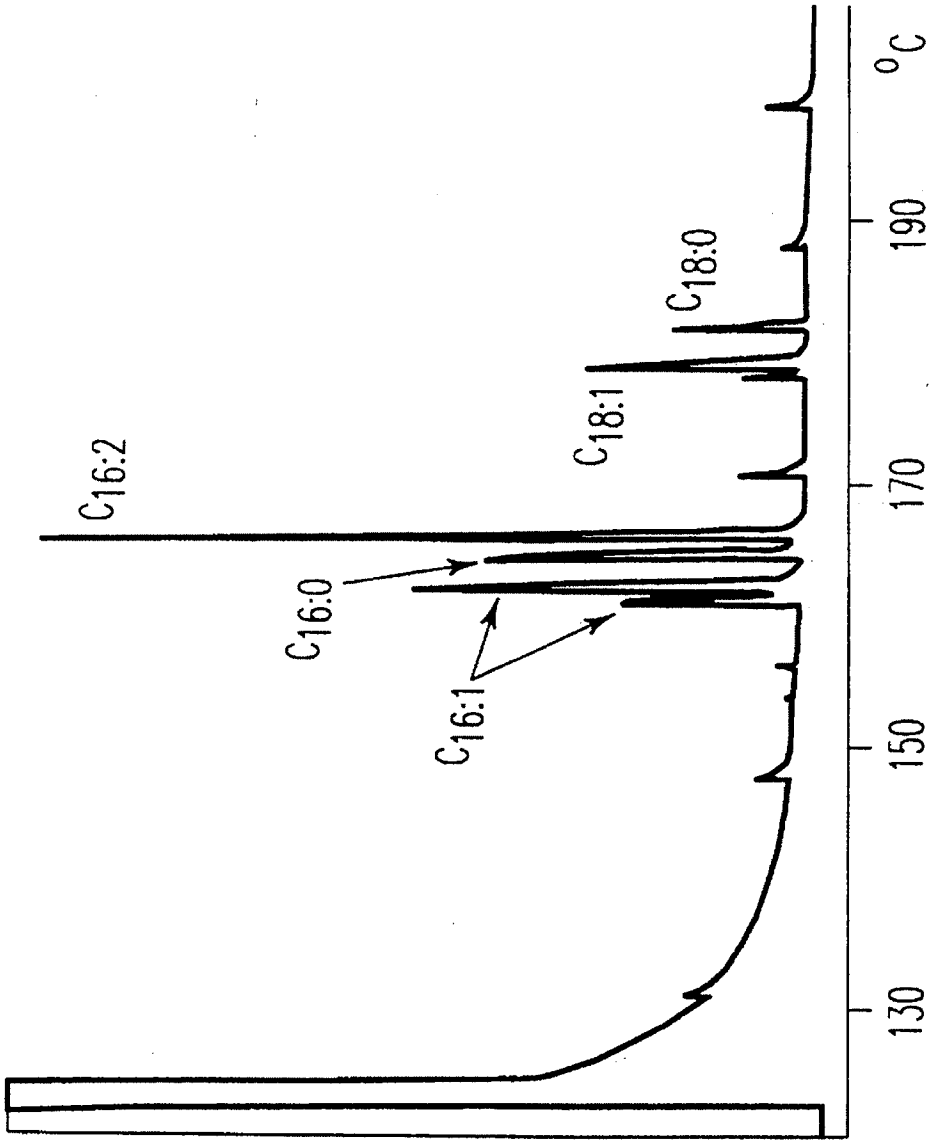


FIG. 2D

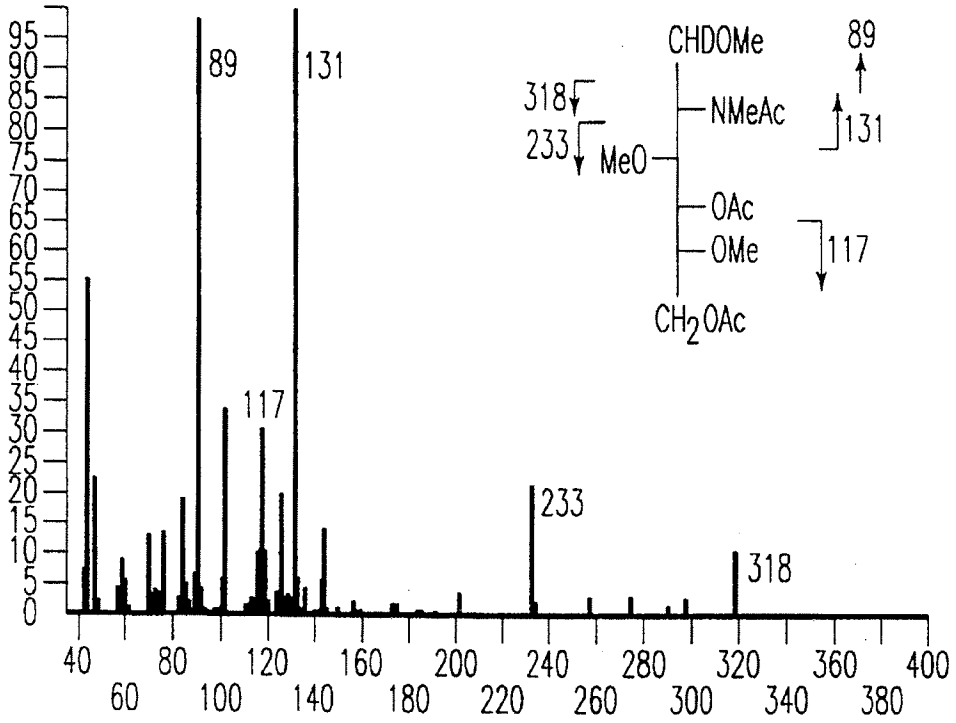

89
131
117
233
318
CHDOMe
NMeAc
MeO
OAc
OMe
CH2OAc
89
131
117
318
233
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400

FIG. 2E

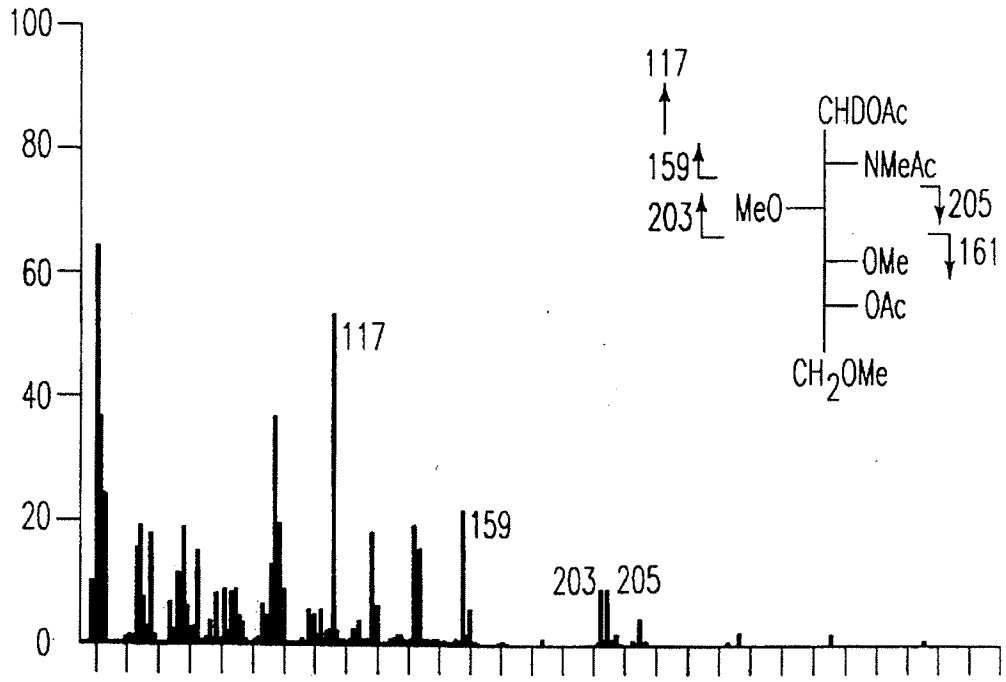

117
159
203
205
161
CHDOAc
NMeAc
MeO
OMe
OAc
CH2OMe
117
159
203
205
100
80
60
40
20
0

FIG. 2F

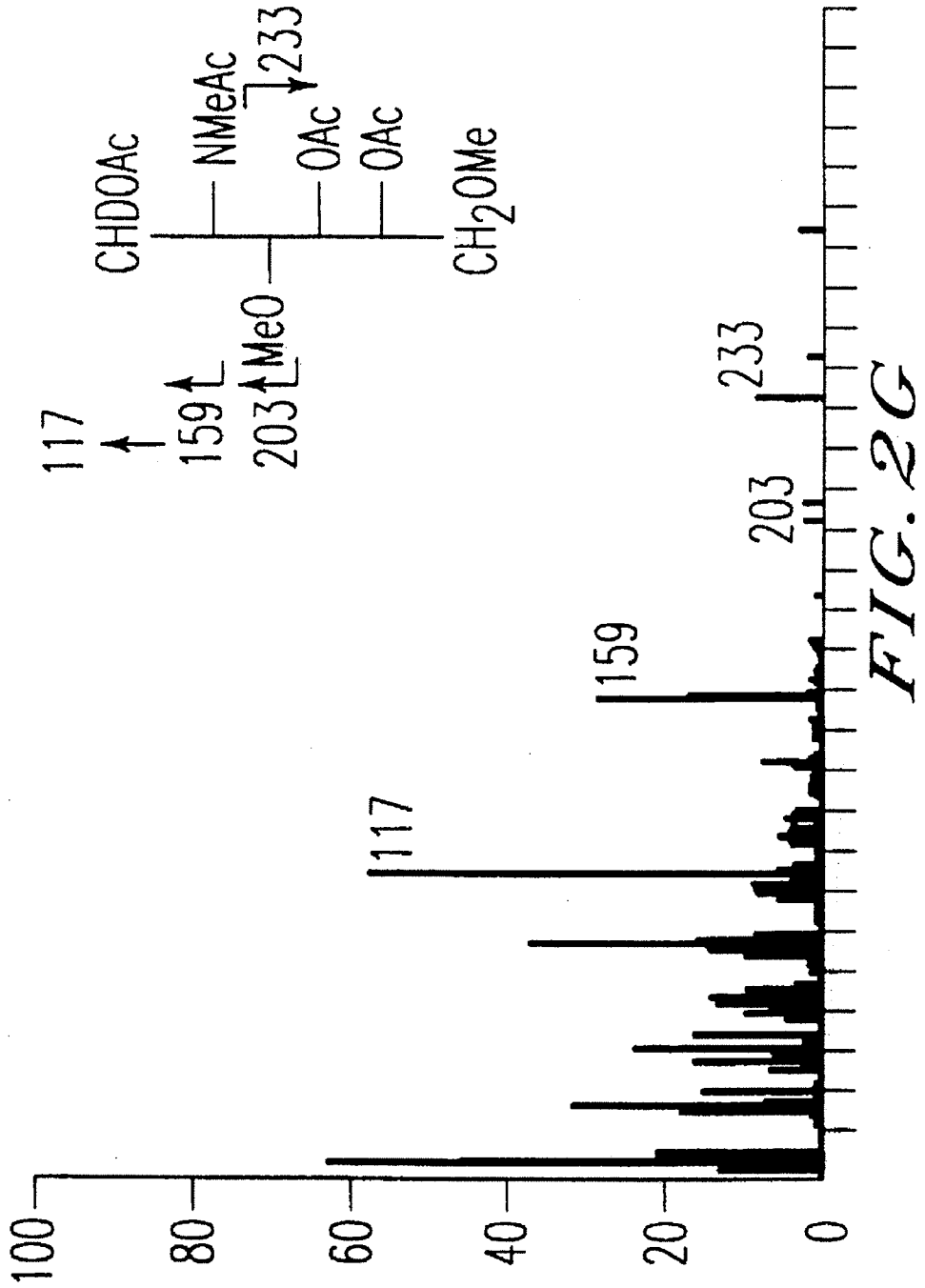

100
80
60
40
20
0
117
159
203
233
CHDOAc
NMeAc
OAc
OAc
CH_2OMe
MeO

FIG. 2G

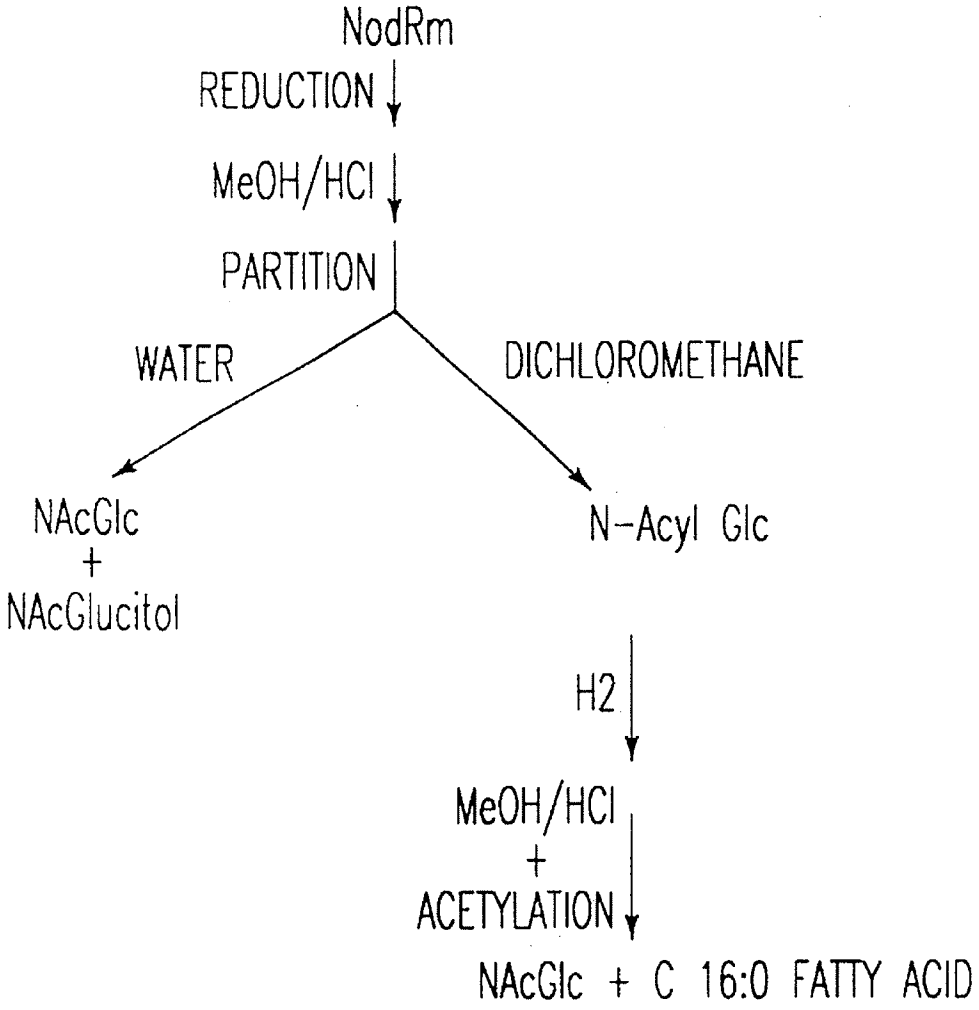

NodRm
REDUCTION
MeOH/HCl
PARTITION
WATER
DICHLOROMETHANE
NAcGlc
+
NAcGlucitol
N-Acyl Glc
H2
MeOH/HCl
+
ACETYLATION
NAcGlc + C 16:0 FATTY ACID

FIG. 3

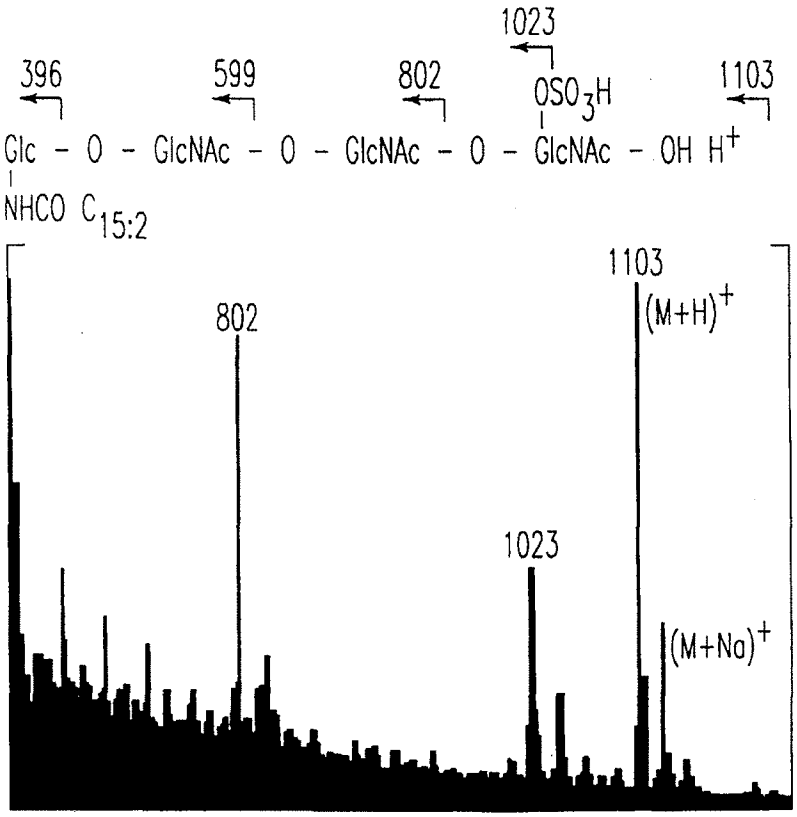

1023
396
599
802
OSO3H
1103
Glc - O - GlcNAc - O - GlcNAc - O - GlcNAc - OH H+
NHCO C15:2
1103
(M+H)+
802
1023
(M+Na)+

FIG. 4A

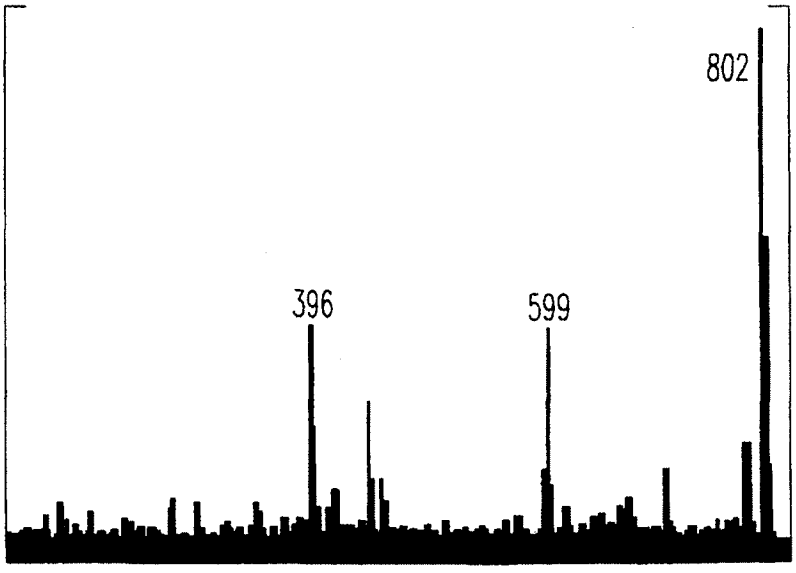

802
396
599

FIG. 4B

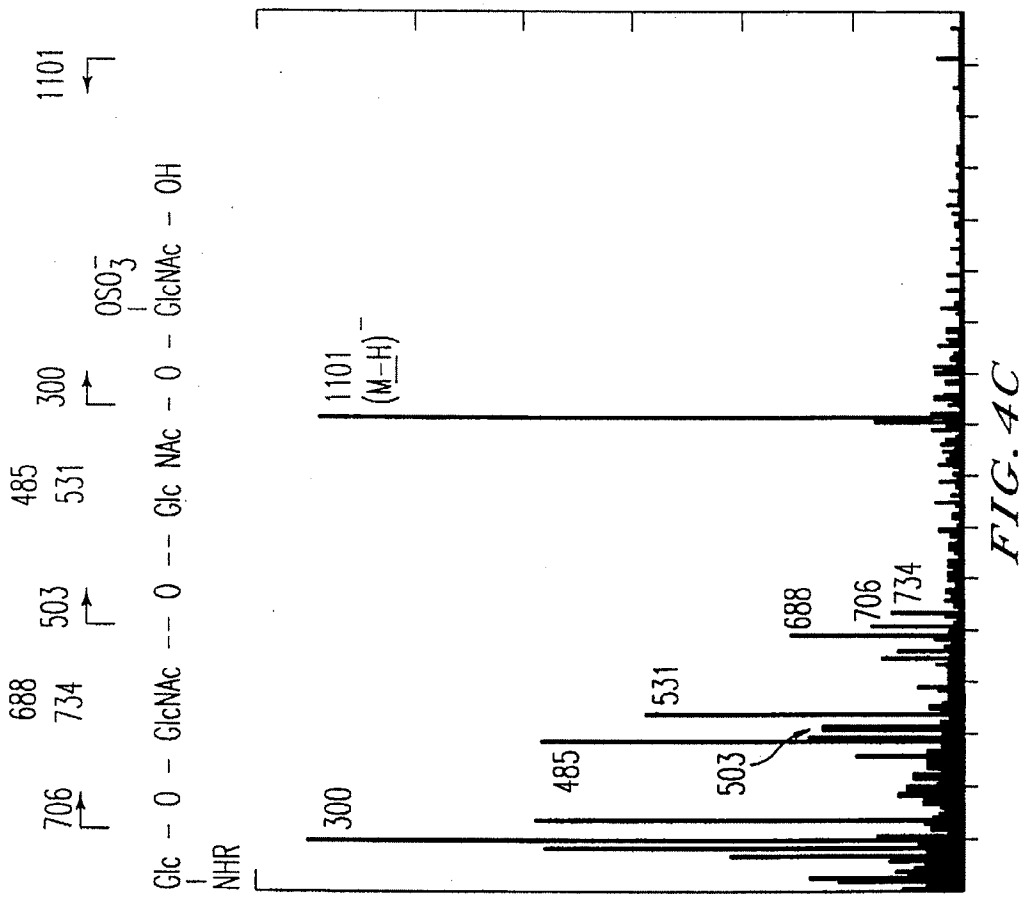

Glc – O – GlcNAc – O – Glc NAc – O – GlcNAc – OH
NHR
OSO3−
706
688
734
503
485
531
300
1101
1101 (M–H)−
300
485
503
531
688
706
734

FIG. 4C

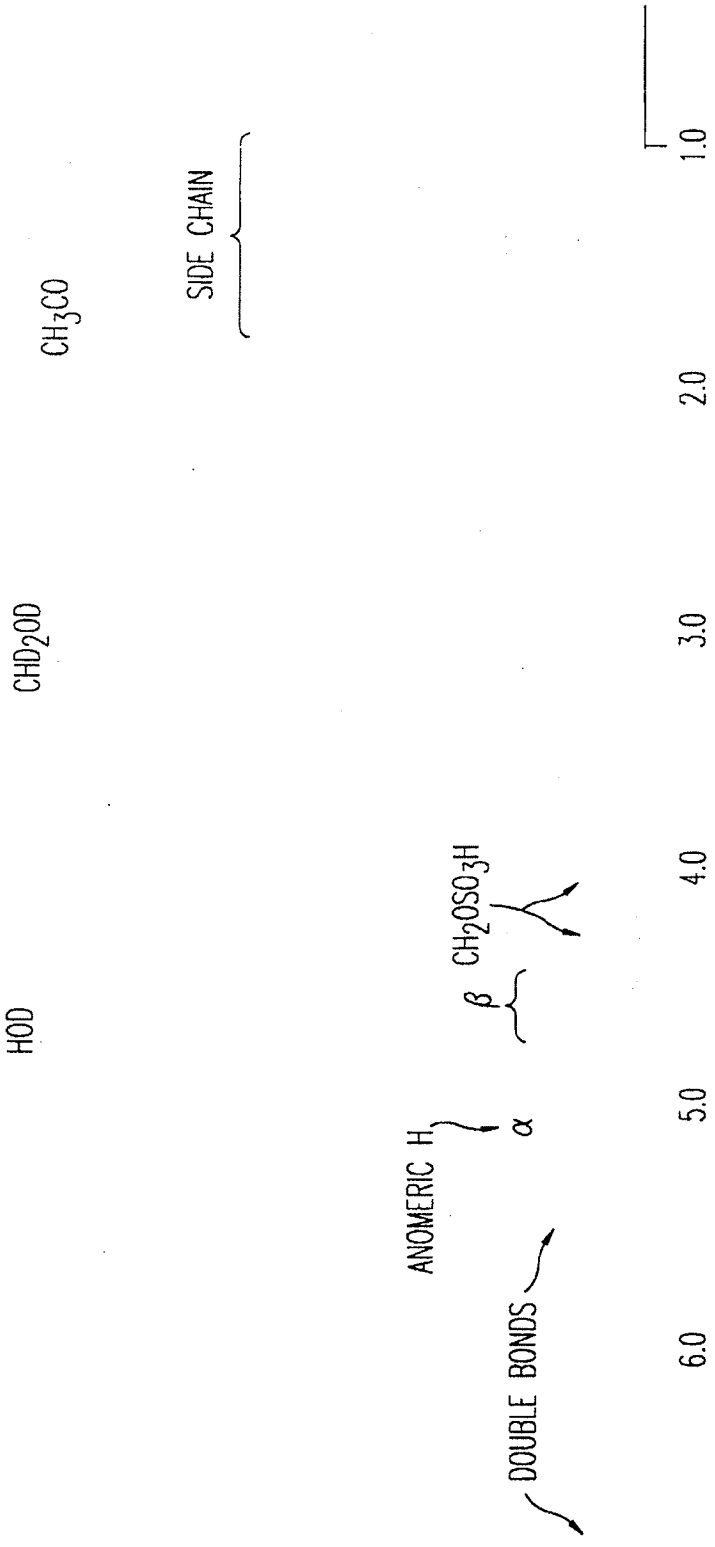

HOD
CHD2OD
CH3CO
SIDE CHAIN
1.0
2.0
3.0
4.0
5.0
6.0
ANOMERIC H
α
β
CH2OSO3H
DOUBLE BONDS

FIG. 5A

FIG. 5B

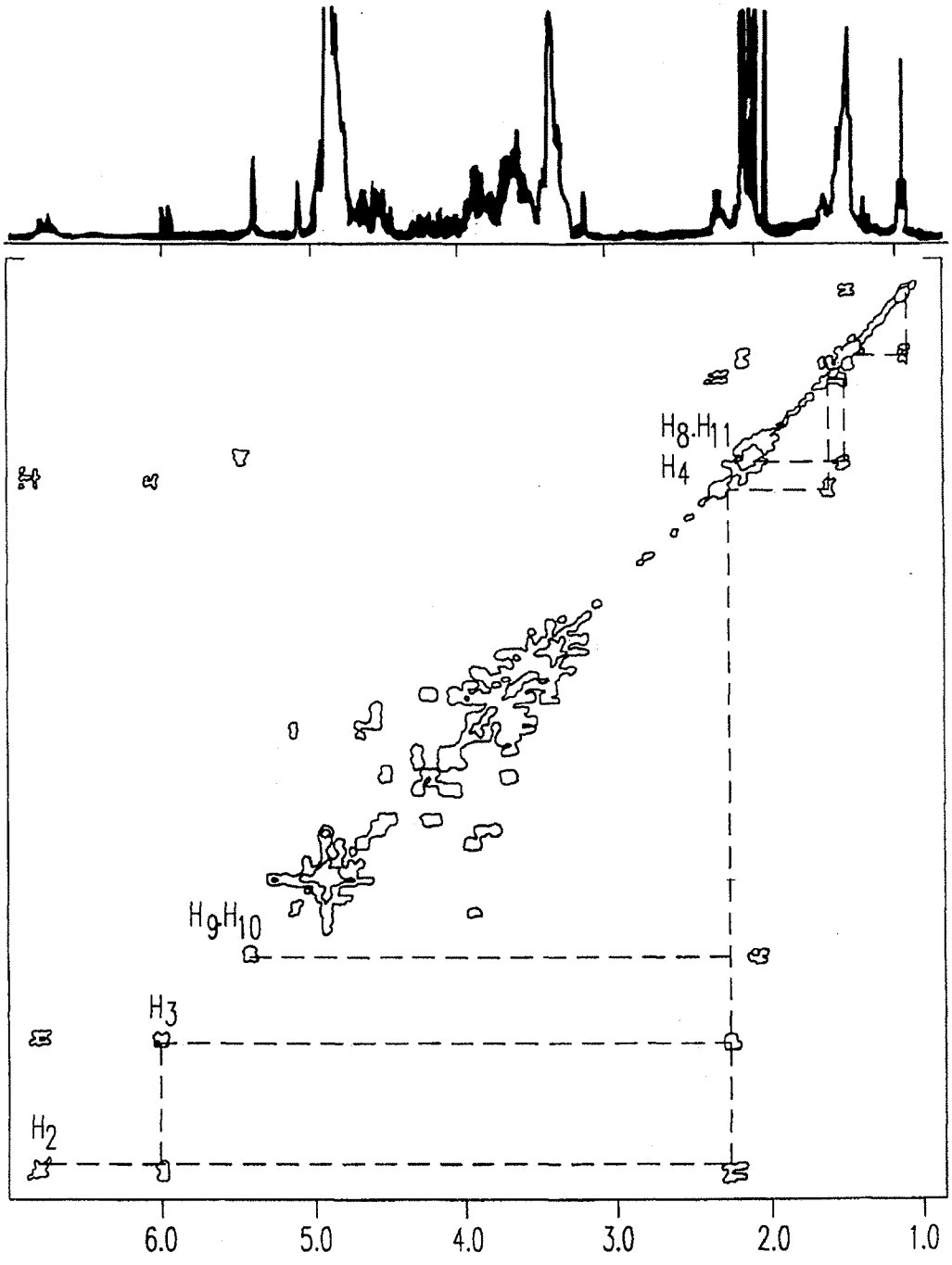


FIG. 5C

FIG. 5D

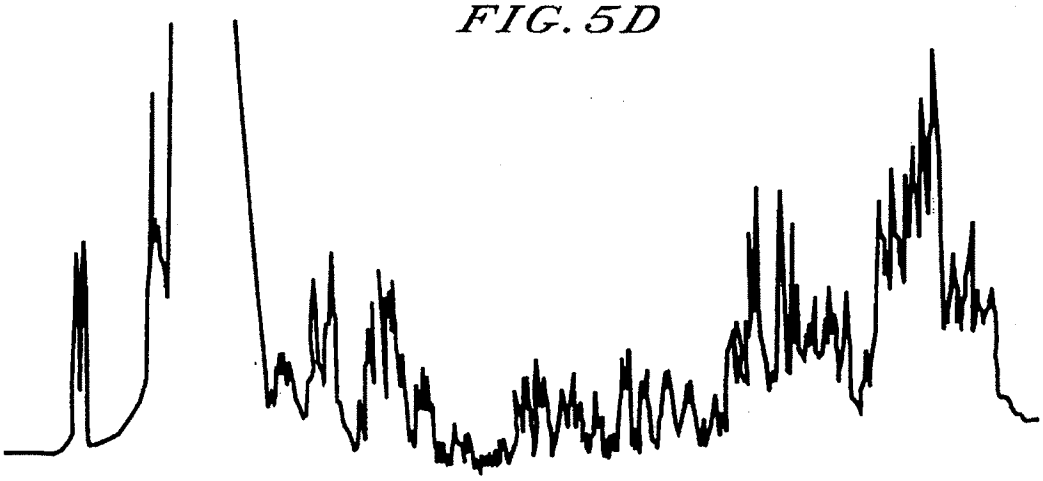
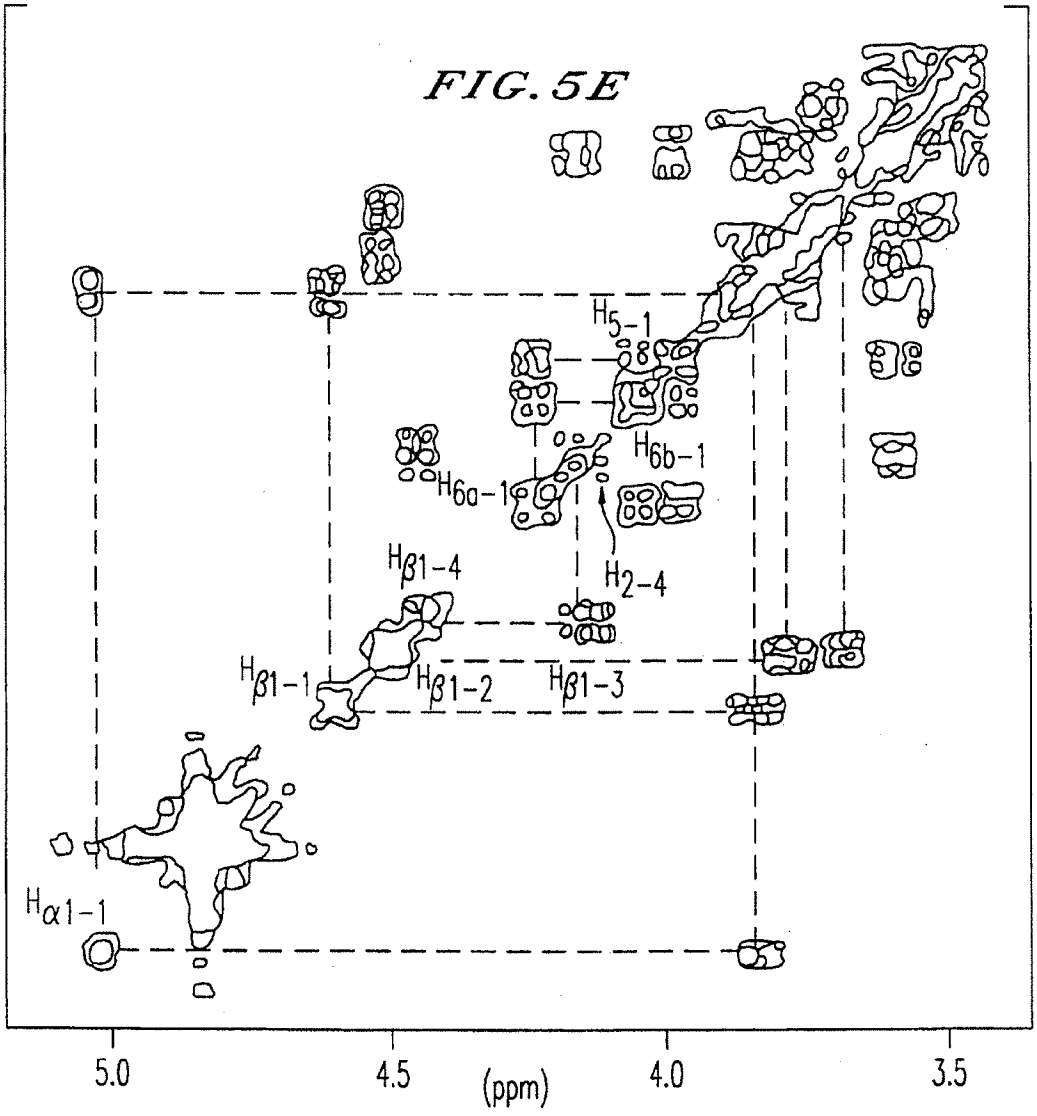


FIG. 5E



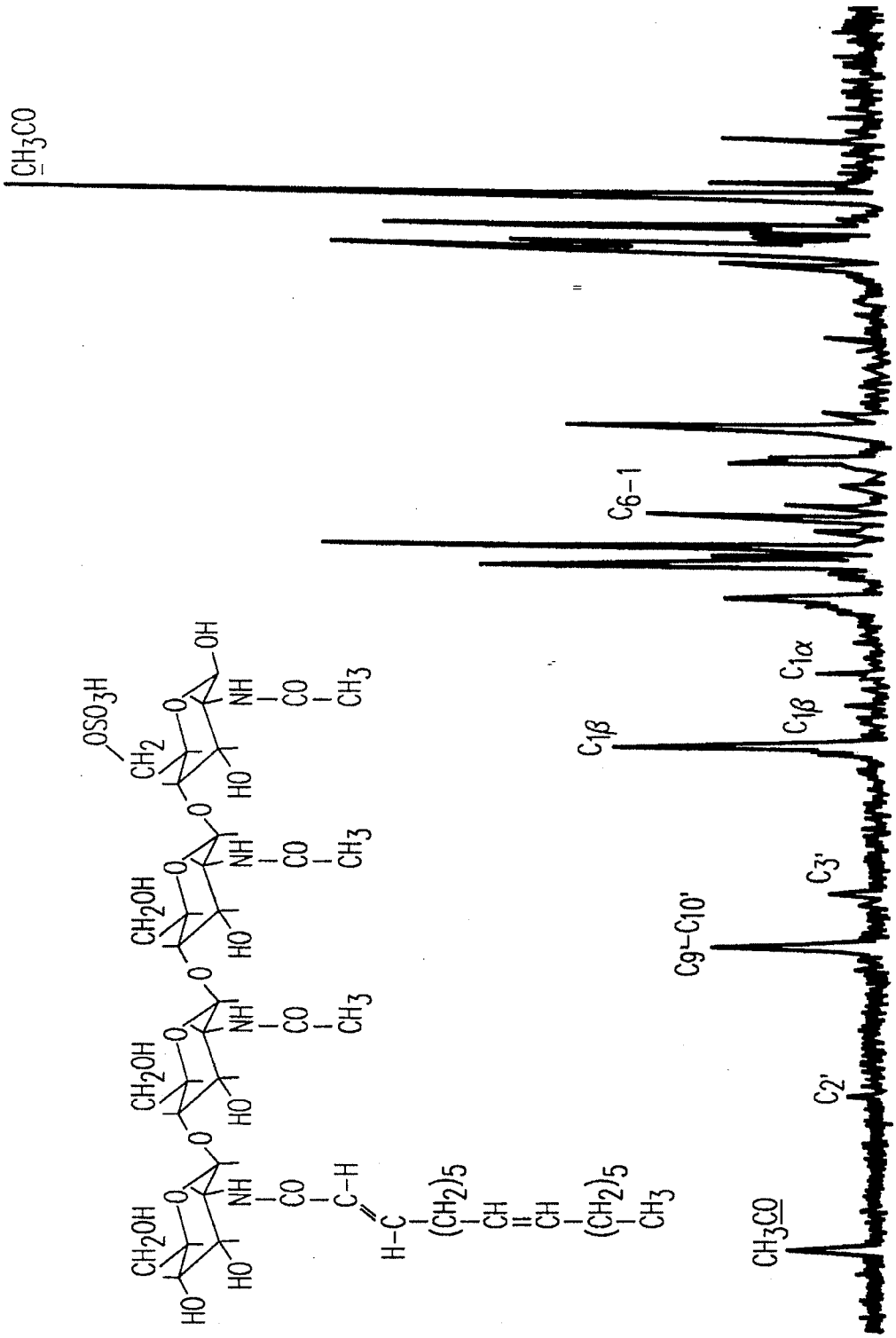

CH3CO
C6-1
C1β
C1β
C1α
C3'
C9-C10'
C2'
CH3CO
OSO3H
CH2
O
OH
NH
CO
CH3
HO
CH2OH
H-C=C-(CH2)5-CH=CH-(CH2)5-CH3

FIG. 5F

FIG. 6A

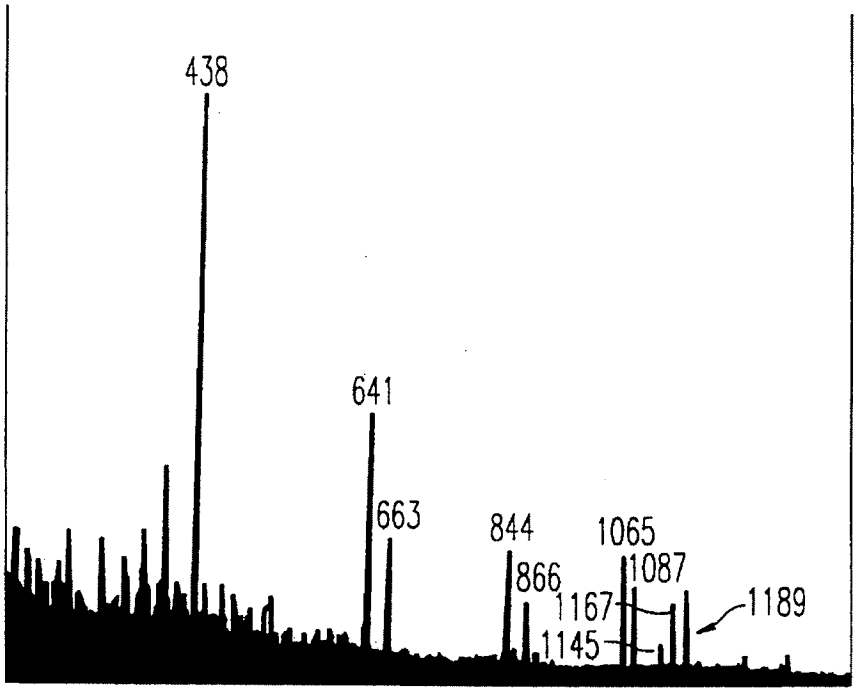
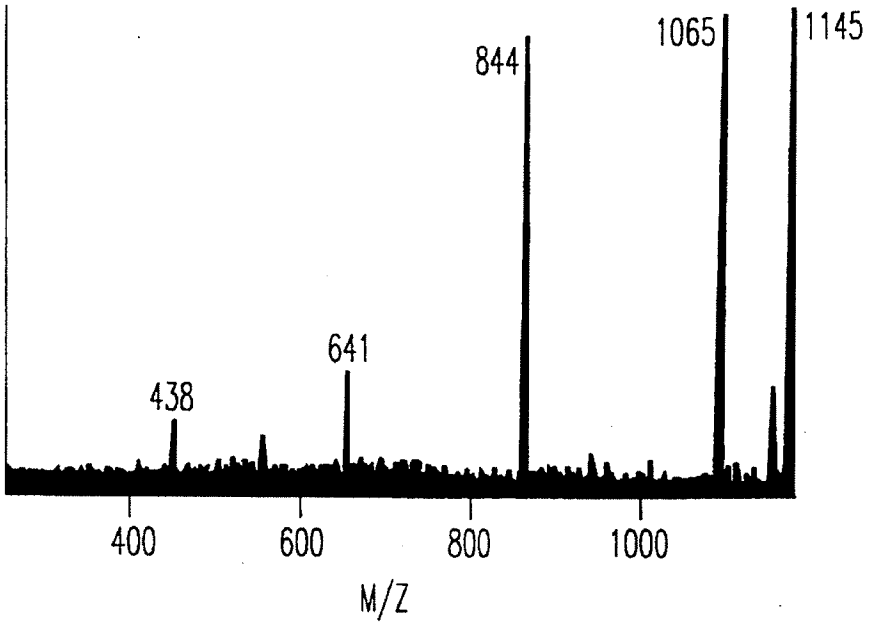

438
641
663
844
866
1065
1087
1167
1145
1189

FIG. 6B


1065
1145
844
641
438
400
600
800
1000
M/Z

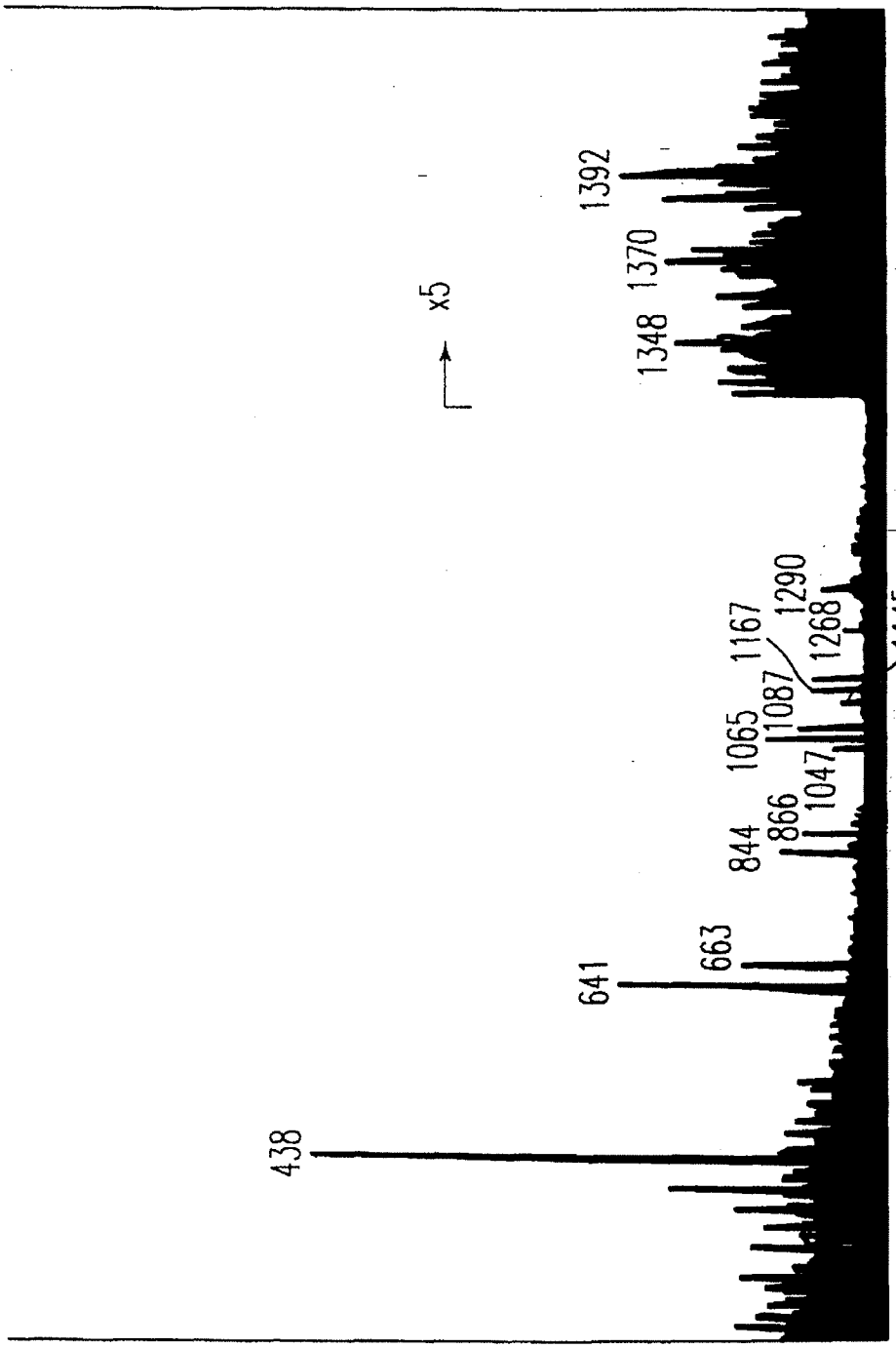

438
641
663
844
866
1047
1065
1087
1167
1268
1290
1145
x5
1348
1370
1392

FIG. 7

SUBSTANCE WITH LIPO-OLIGOSACCHARIDE STRUCTURE CAPABLE OF ACTING AS PLANT-SPECIFIC SYMBIOTIC SIGNALS, PROCESSES FOR PRODUCING THEM AND THEIR APPLICATIONS

This application is a Continuation of application Ser. No. 08/214,676, filed on Mar. 21, 1994, now abandoned, which is a Continuation of application Ser. No. 07/930,662, filed as PCT/FR91/00283, on Apr. 5, 1991, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to substances of lipo-oligosaccharide structure which are capable of forming plant-specific symbiotic signals, to processes for producing these substances and to their applications.

2. Discussion of the Background

As it is known, plants require for their growth a source of combined nitrogen such as ammonia or nitrate. The fixing of nitrogen, by chemical or biological reduction of atmospheric nitrogen (N_2) to ammonia (NH_3), therefore plays a vital role in agricultural production.

As it is also known, symbiotic microorganisms can promote the growth and development of plants through biological fixation of nitrogen.

Rhizobiaceae are Gram negative soil bacteria which generally fix nitrogen in symbiotic association with plants: the establishment of such symbiosis with nitrogen-fixing bacteria allows numerous plant species to grow in soils with low assimilable nitrogen levels. By virtue of photosynthesis, the plant partner provides the bacteria with the energy required for reducing molecular nitrogen to ammonia. In return, the ammonia fixed by the microsymbiont is provided to the host plant which incorporates it into its nitrogen metabolism. The symbiotic association which is established between nitrogen-fixing bacteria such as Rhizobiaceae and plants of the Leguminoseae family is the most important from an ecological and agronomic point of view. This association leads to the formation of nodosities or nodules mainly on the roots of the host plants. Inside these nodosities, the bacteria reduce atmospheric nitrogen to ammonia by means of the nitrogenase enzymatic complex.

The symbiosis between nitrogen-fixing bacteria of the Rhizobiaceae family (Rhizobium, Bradyrhizobium, Sinorhizobium and Azorhizobium genera) and plants of the Leguminoseae family therefore play a very important role in temporate and tropical agriculture. Oil- and protein-rich plants such as soybean and groundnut, fodder plants such as lucerne and clover, protein-rich plants such as peas and field bean, food plants such as beans, peas, lentils and chickpeas, green manures such as Sesbania and the like. By virtue of these symbioses, the cultivation of Leguminoseae is often less costly in nitrogenous fertilizer than the cultivation of plants belonging to other families. In this respect, it should be noted that the massive use of nitrogenous fertilisers has certain disadvantages. Firstly, the synthesis, transportation and application of fertilizers is costly in fossil energy, and this has several consequences: at the farming level it increases the production hosts of the farmers, and at the environmental level it contributes to the greenhouse effect by increasing the CO_2 content. Moreover, an ill thought-out or excessive application of nitrogenous fertilizers causes pollution of fresh waters with eutrophication of the surface

waters and an increase in the nitrate content of the ground water table. These various reasons militate in favor of an increased use of biological fixation of nitrogen.

Given the very damaging consequences of the excessive use of nitrogenous fertilizers, it is therefore necessary to increase the contribution of the biological fixation of nitrogen by plants and in particular by the cultivated species which play an important role in human and animal nutrition. The most acceptable solution both from the ecological and economical point of view is to improve the Rhizobiaceae-Leguminoseae symbiosis.

It has been proposed to carry out this improvement by providing Rhizobiaceae (in particular Rhizobium or *Bradyrhizobium*) at the time of sowing, either by coating the seeds or by means of granules mixed with the seeds or by means of cultivation in liquid medium. These bacteria supplies are however effective only in the relatively rare cases where the appropriate symbiotic bacteria are naturally absent or are not very abundant in the soils. In the opposite cases, that is to say in soils already containing these bacteria, it is practically impossible to impose a strain which is deliberately introduced, due to competition with the indigenous bacteria present in the soils, which, even if they are not necessarily effective for fixing nitrogen, constitute nevertheless a limiting factor for introducing selected bacteria.

It has also been proposed to enhance the Rhizobium-Leguminoseae symbiosis by treating the plants with an exopolysaccharide derived from bacteria of the Rhizobium genus or with an oligosaccharide containing one or more units of such an exopolysaccharide (EPS)—cf. the PCT International Application published under the No. WO 87/06796 filed on behalf of THE AUSTRALIAN NATIONAL UNIVERSITY and mentioning as inventors:. B. G. ROLFE, S. P. DJORDJEVIC, J. W. REDMOND and M. BATLEY. However, this EPS is a product encoded by non-symbiotic genes. In fact, the biosynthesis of these exopoly-saccharides is not under the direct control of the nod genes which control infection and nodulation. These exopolysaccharides are synthesized by Rhizobium strains whose plasmid pSym has been cured, which plasmid carries most of the symbiotic genes and in particular the nod genes.

The genes involved in the nodule-formation process have been localized and several common and specific nodulation genes (nod genes) have been identified and characterized (see Long, S. R., Cell, 1989, 56, 203–214). Whereas the nodA,B,C genes are nodulation genes which are common to the various species of symbiotic Rhizobiaceae, specific nod genes exist which determine the host spectrum and which therefore vary in the various species, and regulatory genes of the nodD type which control the expression of the entire nod genes.

The common genes nodA,B,C have been identified in the four bacterial genera which are capable of establishing a nitrogen-fixing symbiosis with the Leguminoseae: Rhizobium, Bradyrhizobium, Sinorhizobium and Azorhizobium. For the Rhizobium genus, the nucleotide sequeuence of these genes has been obtained in *R. meliloti* (Török et al., *Nucleic Acids Res.*, 1984, 12, 9509–9522; Jacobs et al., *J. Bacteriol.*, 1985, 162, 469–476; Egelhoff et al., DNA, 1985, 4, 241–242). *R leguminosarum* (Rossen et al., Nucl. Acids Res., 1984, 12, 9497–9508), *R. trifolii* (Schofield et al., Nucl. Acids Res:., 1986, 14, 2891–2903).

For *Bradyrhizobium sp.* (Scott, Nucl. Acids Res., 1986, 14, 2905–2919).

For *Azorhizobium caulinodans* (Goethals et al., Mol. Gon. Genet., 1989, 219, 289–298).

A team of researchers comprising several of the inventors of the present invention have shown that in *Rhizobium meliloti*, the common genes nodA,B,C induce, conjointly with the specific genes nodH and nodQ, the production of the host-specific extracellular Nod signals present in the culture supernatants of these bacteria: cf. FAUCHER et al., *J. BACTERIOL* (1988), 172, 5489–5429 and FAUCHER et al., Molec. Plant-Microbe Interact. (1989), 2, 291–300, among others. Furthermore, the latter of these two publications gives an account of the fractionation of the sterile supernatant by ultrafiltration thus making it possible to reveal the presence of two Nod factors with an apparent molecular mass of less than 5,000 Da.

The specificity of infection and nodulation is determined in *R. meliloti* at two levels, namely:—the nodD genes activate the expression of other nod operons depending on the presence of specific signals produced by the plants (Gyorgypal et al., Molec. Gen. Genet., 1988, 212, 85–92) and, —specific genes such as nodH and nodQ determine, when they are activated, the production of bacterial extracellular signals (Nod factors) which make it possible to recognize and stimulate a host leguminous plant such as lucerne (Faucher et al., *J. Bacteriol,* 1988, 172, 5489–5499; Faucher et al., *Molec. Plant-Microbe Interact.,* 1989, 2, 291–300) . However, the chemical structure of the bacterial signals was not known.

SUMMARY OF THE INVENTION

The aim of the present invention is therefore to define the chemical structure of symbiotic Nod signals and to provide substances possessing such a chemical structure and also to provide processes for producing substances which are capable of acting as such symbiotic Nod signals and to propose applications of these substances for the treatment of organisms belonging both to the plant and animal kingdoms (including man).

The subject of the present invention is an essentially pure substance possessing the structure of a Nod factor or of one of its analogs, which Nod factor is characterized by the fact that its biosynthesis is under the control of at least one nodulation gene (nodA,B,C) common to the Rhizobiaceae, in particular to the Rhizobium, Bradyrhizobium, Sinorhizobium and Azorhizobium genera, which substance is characterized in that it consists of a lipo-oligosaccharide not derived from exopolysaccharides.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1A** is a reverse-phase HPLC chromatogram of the compound extracted from *Rhizobium meliloti* as described in Example B.1.2. FIG. **1B** is a gel permeation chromatogram of the compound described in Example B.1.3. FIG. **1C** is an ion-exchange chromatogram of the compound described in Example B.1.4. FIGS. **1D–1E** are reverse phase HPLC chromatograms of the compound described in Example B.1.5.

FIG. **2A** is a vapor phase chromatogram of the reduced compound described in Example B.2.1. FIG. **2B** is a vapor phase chromatogram of the hydrogenated compound described in Example B.2.1. FIG. **2C** is an electron impact mass spectra of the reduced compound described in Example B.2.1. FIG. **2D** is a vapor phase chromatogram of the compound described in Example B.2.2. FIGS. **2E–2G** are vapor phase chromatograms of the compound described in Example B.2.4.

FIG. **3** is a flow chart depicting the three types of glucosamine present in the compound of Example B.2.1.

FIGS. **4A–C** are spectra of the compound described in Example B.3; **4A** is a FAB mass spectrum; **4B** is a MS—MS spectrum; and FIG. **4C** is negative FAB ionization mass spectrum.

FIGS. **5A–5F** are NMR spectra of the compound described in Example B.4. **5A** is a ^1H NMR spectrum; **5B** and **5C** are a COSY ^1H homonuclear 2D-NMR spectra; and **5D–5F** are ^{13}C NMR spectra.

FIGS. **6A** and **6B** are mass spectra as described in Example 2.1.2. **6A** is a positive mode FAB mass spectrum and **6B** is MS/MS spectrum.

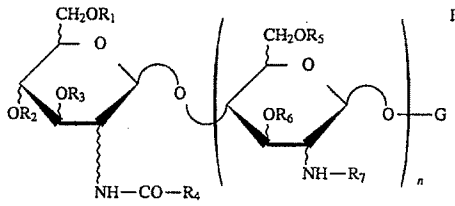
FIG. **7** is positive mode FAB mass spectrum as described in Example 2.2.

Within the context of the present invention, "Nod factor" is understood to mean a signal molecule produced under the direct control of nod genes, by means of which signal the symbiotic bacteria are capable of infecting plants and inducing the formation of nodosities.

According to an advantageous embodiment of the said essentially pure substance, the Nod factor whose structure it possesses is characterized in that it is a plant-specific symbiotic signal and is capable of enhancing the capacity of the bacteria to infect the host plant with which it is associated and/or of accelerating the formation of nodules on the host plant with which it is associated and/or of inducing the transcription of the symbiotic genes of Leguminoseae.

According to another advantageous embodiment of the said essentially pure substance conforming to the invention, the Nod factor whose structure it possesses has the structural properties of a lectin ligand.

The subject of the present invention is also a lipo-oligosaccharide substance characterized in that it is of the general formula I below:



FORMULA I

in which:

G is a hexosamine which is variously substituted, for example, by an acetyl group on the nitrogen, a sulfate group, an acetyl group and/or an ether group on an oxygen,

R_1, R_2, R_3, R_5, R_6 and R_7, which may be identical or different, represent H, CH_3CO—, C_xH_yCO— where x is an integer between 0 and 17, and y is an integer between 1 and 35, or any other acyl group such as for example a carbamyl

R_4 represents a mono-, di- or triunsaturated aliphatic chain containing at least 12 carbon atoms, and

n is an integer between 1 and 4.

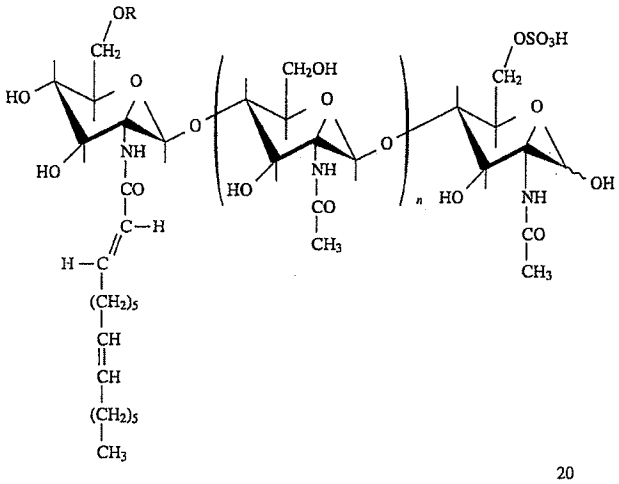
According to a preferred embodiment of the present invention, G represents:

in *R. meliloti*, an N-acetyl-D-glucosamine 6-sulfate

in R. leguminosarum b.v. viciae, an N-acetyl-D-glucosamine.

According to an advantageous form of this embodiment, the lipo-oligosaccharide conforming to the invention is characterized in that it is of the formula (II) below:

FORMULA II



in which R represents H or CH_3CO— and n is equal to 2 or 3.

The *R. meliloti* lipo-oligosaccharides of general formula (II), in which R represents H, are designated below by the general term NodRm; when n=2, the corresponding lipo-oligosaccharide is called NodRm-1; when n=3, the corresponding lipo-oligosaccharide is called NodRm-3.

The lipo-oligosaccharides of general formula (II), in which R represents CH_3CO—, are designated below by the general term Ac-NodRm; when n=2, the corresponding lipo-oligosaccharide is called Ac-NodRm-1; when n=3, the corresponding lipo-oligosaccharide is called Ac-NodRm-3.

The aim of the present invention is also to provide processes for producing the essentially pure substances conforming to the present invention, the said processes include purification processes using Rhizobiaceae culture supernatants as starting material, routine processes using sequential or convergent synthesis in oside syntheses and processes for producing said substances by genetic engineering using as starting material the nod genes cloned into microorganisms belonging to the Rhizobiaceae family or otherwise, optionally under the control of an appropriate promoter and/or in the presence of regulatory genes which have been subjected to appropriate mutations.

According to an advantageous embodiment of a process for producing the essentially pure substance conforming to the present invention, during a first stage, a recombinant plasmid is produced which results from the cloning, into a plasmid capable of replicating in a Rhizobiaceae bacterium or any other appropriate bacterium, (1) either of a fragment which contains common and specific nod genes as well as the regulatory genes of a given Rhizobiaceae bacterium, or (2) of the regulatory genes for the express ion of the nod genes. The Rhizobiaceae bacteria or any other appropriate bacteria are then modified by introducing the said recombinant plasmid in order to obtain a mutant strain which highly overproduces Nod factors and the said mutant strain is cultured in an appropriate culture medium; during a second stage, the culture supernatant is recovered and it is purified by extraction with an appropriate lower alcohol, or by solid-liquid extraction followed by a reversed-phase HPLC chromatography of the extraction residue, a gel permeation chromatography, an ion-exchange chromatography and a reversed-phase analytical HPLC chromatography.

According to an advantageous embodiment of this process, the Nod factor-overproducing plasmid is introduced into a non-exopolysaccharide-producing mutant Rhizobiaceae bacterium.

According to another advantageous embodiment of the process for producing the substantially pure substance conforming to the present invention, a wild strain of Rhizobiaceae bacteria, which are highly productive of Nod factors, is used as starting material, the said strain being cultured in an appropriate culture medium, after which the culture supernatant is recovered and treated using the methods indicated above.

The subject of the present invention is also a plant-treatment agent, the or an active constituent of which is an essentially pure substance as defined above, and in particular:

an agent for stimulating the mechanisms for defending the plants against pathogens,

an agent for stimulating the symbiotic properties of Leguminoseae, especially with respect to nitrogen fixation.

According to an advantageous embodiment of the plant-treatment agent conforming to the present invention, it is formulated in the form of a composition for coating seeds or an aqueous solution or suspension for spraying, in which the said substance is present alone or combined with other active constituents.

According to another advantageous embodiment of the plant-treatment agent conforming to the present invention, the said substance is present in the coating compositions or the aqueous solutions or suspensions at a concentration of between 10^{-6}M and 10^{-14}M when the plant-treatment agent is intended to be used as agent for stimulating the defence mechanisms or the symbiotic properties.

Furthermore, the subject of the present invention is a therapeutic agent, the or an active constituent of which is an essentially pure substance as defined above.

According to an advantageous embodiment of this therapeutic agent, the said substance is present in the therapeutic agent at a concentration of between 10^{-5}M and 10^{-8}M.

In addition to the above provisions, the invention also comprises other provisions which will become apparent from the description below.

The invention will be understood more clearly with the aid of the additional description below which refers to the examples below.

EXAMPLES

It should be clearly understood, however, that these examples, as well as the appended drawings, are given solely as illustration of the subject of the invention and do not constitute in any manner a limitation thereof.

EXAMPLES

Example 1 - Preparation of the compounds conforming to the invention by purification using as starting material a *Rhizobium meliloti* culture medium.

A. Production of the compound

The production of extracellular Nod factors by Rhizobium is under the control, on the one hand, of the common genes nodA,B,C (van Brussel et al., J. Bacteriol., 1986, 165, 517–522; Zaat et al., J. Bacteriol., 1987, 169, 3388–3391; Faucher et al., J. Bacteriol., 1988, 170, 5489–5499) and, on the other hand, of the host-specific nod genes, for example *R. meliloti*, the nodH and nodQ genes (Faucher et al., J. Bacteriol., 1988, 170, 5489–5499; Faucher et al., Molec. Plant-Microbe Interact., 1989, 2, 291–300).

Regulation of the transcription of the common genes nodA,B,C is under the control of the regulatory proteins encoded by the nodD1, nodD2, nodD3 and syrM genes (Mulligan et al., PNAS, 1985, 82, 6609–6613; Gyorgypal et al., Mol. Gen. Genet. , 1988, 212, 85–92) . The protein NodD1 is active only in the presence of certain inductive flavonoids such as luteolin, which are present in the root exudates of Leguminoseae (Mulligan et al., PNAS, 1985, 82., 6609–6613; Peters et al., Science, 1986, 233, 977–980). The presence of the nodD3 and syrM genes in the multicopy plasmid brings about a constitutive activation of the nodA, B,C genes even in the absence of inductive flavonoids (Mulligan et al., Genetics, 1989, 122, 7–18).

To determine the regulation of the other nod genes, the inventors constructed fusions between the genes for host specificity nodE, nodG and nodH and the lacZ gene from E. coli which encodes for β-galactosidase. These fusions made it possible to show that in *R. meliloti*, the nod genes for host specificity are regulated in the same way as the common genes nodA,B,C: (i) their transcription is activated by nodD1, nodD3 and syrM, (ii) luteolin is required for activation by nodD1, (iii) the activation of transcription is much higher when the regulatory genes and the structural genes are in a plasmid of the incompatibility group Inc-P1 (present at 5–10 copies per cell).

This information prompted the inventors to choose a plasmid pRK290, derived from pRK2, in which a 30-kb fragment of the nod region of the *R. meliloti* megaplasmid pSym was cloned. This recombinant plasmid, pGMI149, contains the entire common and specific nod genes as well as the regulatory genes nodD1, nodD3 and syrM (Debellé et al., J. Bacteriol., 1986, 168, 1075–1086; Faucher et al., J. Bacteriol., 1988, 170, 5489–5499). They have shown that *R. meliloti* strains which contain the plasmid pGMI149 overproduce Nod factors: indeed, their production is at least one hundred times greater compared with wild *R. meliloti* strains.

For large scale production of Nod factors, for example for fermenter cultures, the high production of exopolysaccharides can present disadvantages for the filtration of the cultures and for the purification of the signal molecules. The inventors therefore thought it preferable to introduce the overproducing plasmid pGMI149 into the *R. meliloti* mutant EJ355 which does not produce exopolysaccharides. The strain EJ355 (pGMI149) produces Nod factors in abundance but no exopolysaccharides.

By using gene fusions between the nod genes from *R. meliloti* and the lacZ gene from *Escherichia coli* (nod::lac fusions) to measure the level of expression of the nod genes, the inventors developed a culture medium which permits good expression of the nod genes and a high production of Nod factors. A satisfactory medium is an inorganic medium which contains succinate as carbon source and glutamate as nitrogen and carbon source. Indeed, in a rich medium, containing protein lysates and yeast extracts, the activation of the transcription of the nod genes is lower; furthermore, the purification of Nod factors is easier using culture supernatants prepared from simple media. The composition of this medium is for example as follows:

inorganic salts: 14.7 mM KH_2PO_4; 11.5 mM K_2HPO_4; 1 mM $MgSO_4$; 0.46 mM $CaCl_2$; 37 μM $FeCl_3$,

organic compounds: 5.3 mM sodium L-glutamate; 7.4 mM sodium succinate; 2 μM biotin; 10 μM luteolin.

B.1. Purification of the compound

B.1.1. The compound is extracted from the *Rhizobium meliloti* culture medium (15 l) using butanol (2 extractions with 3 liters of BuOH). The butanolic phase is washed with water (1 liter) and then concentrated under vacuum. The residue obtained is dissolved in water (500 ml) and extracted 2 times with ethyl acetate (2 times 300 ml). The aqueous phase is finally concentrated and then freeze-dried.

B.1.2. Reversed-phase HPLC chromatography

The residue obtained by butanolic extraction is chromatographed by reversed-phase HPLC on a 10 μm μ-Bondapak C_{18} column—Water Associates—7.5×250 mm, with a flow rate of 2 ml/min and using a linear gradient from an 80-20 water-ethanol mixture A to pure ethanol B (FIG. **1.1**); the detection is carried out at 220 nm. The area presenting biological activity is collected for further purifications.

B.1.3. Gel permeation chromatography

The fraction is purified by gel permeation on a Sephadex LH20 column (1×20 cm column; LH20 phase, Pharmacia) in a solvent such as ethanol, at a flow rate of 4 ml/hour. The detection is carried out at 220 nm. The column was calibrated beforehand with respect to polyethylene glycol (PEG) standards (cf. FIG. **1.2**). The active shaded fraction is collected.

B.1.4. Ion-exchange chromatography

The active compound (shaded area) is eluted on an ion-exchange column (1×2 cm DEAE-Trisacryl column; IBF) with a linear NaCl gradient between 0 and 0.1N in a 5×10^{-3}N Tris-HCl buffer, pH=8.2 (FIG. **1.3**).

B.1.5. Reversed-phase analytical HPLC chromatography

The final purification step is a reversed-phase HPLC chromatography on an analytical column (4.5×250 mm; 5 μm Spherisorb/C_{18} phase) with an 80-20 water-acetonitrile mixture as solvent at a flow rate of 1 ml/min; 300 μg are injected (FIG. **1.4***a*). Two peaks absorbing at 220 nm are thus detected and exhibit biological activity. Structural study will show that it is in fact the same compound appearing in two forms in equilibrium (free α and β anomeric forms).

B.2. Analysis of the constituents

B.2.1. Analysis of the monosaccharides

After complete hydrolysis of the compound (3N HCl; 3 h; 100° C.) and then derivatization to peracetylated methyl glycoside, a single monosaccharide, identified as being 2-deoxy-2-acetamidoglucose or acetyl glucosamine (NAc Glc), is observed by vapor-phase chromatography. This sugar was shown to be of the D series after preparation of the glucoside peracetate with a chiral alcohol and comparison, using VPC, with (–)-2-butanol-N-acetylglucosamine standards of the D and L series derived in a similar manner.

Reduction of the compound with sodium borodeuteride (1N $NaBD_4/H_2O$; 18 h; 20° C.) is followed by controlled methanolysis (1N MeOH/HCl; 80° C.; 1 h) and then by a water-dichloromethane partition. The aqueous phase contains 1-0-Me-NacGln and 2-deoxy-2-acetamidoglucitol which are identified by VPC (FIG. **2.1**); identification by VPC is carried out on a 0.32 mm×30 m OV1 column using helium and an FID detector. The organic phase contains the

methyl glycoside of the glucosamine which is N-acylated by a $C_{16:2}$ fatty acid and which is identified by its electron impact mass spectrum on the pertrimethylsilylated derivative using data from the literature (Demary, M. et al., Nouv. J. Chimie, 1977, 2, 373–378); the electron impact mass spectrum (EI-MS) of the 1-OMe glucosamine, which is amidated on the nitrogen atom by a fatty acid and pertrimethylsilylated, is represented in FIG. **2.3**. After catalytic hydrogenation of the side chain, methanolysis (MeOH, 3N HCl; 2 h; 100° C.) and then acetylation, VPC analysis—carried out on a 0.32 mm×30 m OV1 column using helium and an FID detector—of the peracetylated derivatives makes it possible to confirm the structure of this N-acyl-sugar by visualizing 1-OMe-GlcNAc and methyl palmitate (accompanied by methyl stearate in a smaller quantity) (FIG. **2.2**).

This analysis makes it possible to establish the presence of three types of glucosamine: NAcGlc, reducing NacGlc and N-acyl Glc, using an analytical procedure summarized in FIG. **3** which is appended.

B.2.2. Fatty acid composition

It was possible for the fatty acids present in the compound to be isolated after saponification (5% KOH; 18 h; 80° C.) and then analyzed by VPC—on a 0.32 mm×30 m OV1 column using helium and an FID detector—in the form of methyl esters (FIG. **2.4**). This study made it possible to identify, in addition to a predominant $C_{16:2}$ fatty acid, other minor entities: $C_{16:0}$, $C_{16:1}$, $C_{18:0}$ and $C_{18:1}$. It was possible for the position of the two double bonds of the $C_{16:2}$ fatty acid to be specified from the MS—MS mass spectrum of the carboxylate formed from its perfluorobenzyl ester (J. C. Promé et al., Rapid Comm. Mass Spectrom., 1987, 1, 80–82) and localized in 2 and 9 of the chain.

B.2.3. Detection of a sulfate functional group

After culturing the bacteria in the presence of ^{35}S-labeled sodium sulfate and then purification of the compound, the observed correlation between the UV-absorption profile and ^{35}S-incorporation makes it possible to establish the presence of a sulfate functional group in this compound. FIG. **1.4***b* represents the said correlation which is obtained by incorporation of ^{35}S, after culturing on (^{35}S)-Na_2SO_4 and then fractionating on the basis of the profile represented in FIG. **1.4***a* and counting the radioactivity by liquid scintillation.

B.2.4. Determination of the mode of interglycoside bonding

The mode of bonding between the various glucosamines was established after permethylation of the $NaBD_4$-reduced compound, hydrolysis and preparation of the alditol acetates according to previously-described methods (K. Stellner et al., Arch. Biochem. Biophys., 1973, 155, 464–472), followed by a VPC-MS analysis; the electron impact mass spectrum of the alditol acetates obtained from permethylation of the reduced compound is represented in FIG. **2.5**. This study made it possible to identify alditol acetates which are tetramethylated in positions 1, 2, 3 and 5 (spectrum a) and 2, 3, 4 and 6 (spectrum b) and tri-O-methylated in positions 2, 3 and 6 (spectrum c), derived respectively from a reducing glucosamine which is substituted in positions 4 and 6, a terminal glucosamine and a glucosamine which is bonded in positions 1 and 4.

B.3. Mass spectrometry

B.3.1. Positive mode FAB mass spectrometry

The positive mode FAB mass spectrum of the compound (FIG. **4.1**, scanning from m/z 4,000 to m/z 100 (nominal mass)) shows a pseudomolecular ion $(M+H)^+$, m/z=1103, accompanied by a sodium-containing ion corresponding to m/z=1125. Fragment ions are also observed at m/z=1023 and 802, which are attributed to sulfate and NAcGlucosamine sulfate losses respectively. Decomposition of the m/z=802 ion is observed by MS—MS spectrometry (FIG. **4.2**; B/E scan) and leads to two glycosilyl fragments at m/z=599 and 396 following the breaks between the various monosaccharides. These two spectra make it possible to establish a linear tetrasaccharide structure whose reducing sugar is substituted by a sulfate functional group, and the terminal sugar by a $C_{16:2}$ fatty acid.

B.3.2. Negative mode FAB mass spectrometry

Apart from the pseudomolecular ion $(M-H)^-$ at m/z=1101, the negative mode FAB spectrum exhibits fragments which are attributable to the glycoside cavities. Analysis of these ions leads to structural conclusions similar to those described above: cf. FIG. **4.3** which shows the negative FAB ionisation mass spectrum, using thioglycerol as matrix.

B.4. NMR

B.4.1. ^1H NMR and proton homonuclear 2D-NMR (COSY)

The data relating to the ^1H NMR and COSY-NMR spectra make it possible to establish the following structural elements:

a) The side chain exhibits in ^1H NMR two types of vinyl protons corresponding to the two double bonds. Two low-field signals (δ=5.95 ppm; 1 H and δ=6.80 ppm; 1 H) are attributed to a double bond conjugated to the carbonyl functional group and they possess a large coupling constant (J=15 Hz) characteristic of an E configuration; FIG. **5.1** represents the abovementioned ^1H NMR spectrum; CD_3OD is used as solvent and a frequency of 300 MHz is used ("Bruker" apparatus). The two magnetically equivalent protons of the double bond inside the chain appear as a single signal (δ=5.35 ppm). The protons of these two double bonds exhibit, in 2D-COSY, a series of correlations with the other aliphatic protons which is characteristic of a linear chain; FIG. **5.2** represents the COSY ^1H homonuclear 2D-NMR spectrum obtained using CD_3OD as solvent and a frequency of 300 MHz ("Bruker" apparatus).

b) Anomeric protons: the COSY ^1H homonuclear 2D-NMR spectrum of the saccharide region is represented in FIG. **5.3**; this spectrum, which was obtained using CD_3OD as solvent and a frequency of 300 MHz ("Bruker" apparatus), shows, in the 3.4 to 5.2 ppm saccharide proton region, 5 signals having only one correlation, which are attributed to the anomeric protons. Furthermore, no signal is masked by the unresolved complex for water, as indicated by the lack of correlation in this region of the spectrum. These anomeric signals correspond to three doublets of the β bonds (δ=4.40 to 4.55 ppm; 3 H; J=8.5 Hz) and two doublets attributed to the anomeric protons of the α and β forms (δ(α)=5.05 ppm; J=3.4 Hz and δ(β)=4.60 ppm; J=8.5 Hz).

c) Protons of the rings: the saccharide rings are characterized by the methyl signals of the acetamide groups, which are split by the coexistence of the free α and β forms of the oligosaccharide (δ=2.0 to 2.15 ppm; 6s; 9H). The other protons of the ring resonate between 3.2 and 3.9 ppm. Only three signals, which are more unmasked, are attributed to the hydrogens situated near the sulfate group. The correlation mode observed by 2D-COSY (FIG. **5.3**) between these three signals is characteristic of protons 5 and 6 (H5/H6b, H5/H6a and H6a/H6b correlation). This result makes it possible to place the sulfate functional group in position 6.

B.4.2. ^{13}C NMR

Attribution of the various signals of the proton-decoupled ^{13}C NMR spectrum (FIG. **5.4**) made it possible to confirm the presence of a di-unsaturated fatty acid chain, the β1→4 mode of bonding between the glucosamines as well as the position of the sulphate in 6 from the following elements: FIG. **5.4** represents the ^{13}C NMR spectrum obtained at 50.4 MHz ("bruker" apparatus) using D_2O as solvent:

a) Fatty acid chain: the chemical shifts of the carbons of the side chain ($C_{2'}$ to $C_{16'}$) are characteristic of a fatty acid possessing a conjugated double bond ($C_{2'}$ and $C_{3'}$; δ=125 and 155 ppm) and an inner double bond ($C_{9'}$ and $C_{10'}$; δ=133 ppm).

b) Anomeric carbons: a preponderant signal (δ=103.7 ppm) which is attributed to the anomeric carbons of the nonreducing monosaccharides exhibits a chemical shift which is similar to the C_1 of the methyl glucopyranoside of NAcGlc. Two signals, of lower intensities, are attributed to the C_1 of the reducing glucosamine appearing in these two free anomeric forms ($\delta C_1\alpha$=93.1 ppm and $\delta C_1\beta$=97.8 ppm).

c) Ring: from the literature data, attribution of the various signals leads to confirmation of the β1→4 linkage in the tetrasaccharide. This attribution is complicated by the separation, into two groups of signals, of the carbons of the reducing glucosamine. The presence of the sulfate functional group in position 6 is supported by the observed unmasking of carbon 6 which carries this functional group ($\delta C_{6\text{-}1}$=68.7 ppm).

Example 2 - Detection of the biological activity of the NodRm-1 factor

The biological activities detected (i) by the specific activity test of deformation of the root hairs (Had) on vetch, described by Zaat et al., (J. Bacteriol., 1987, 169, 3388–3391), (ii) and by the tests of deformation and ramification of lucerne root hairs, described by Faucher et al. (J. Bacteriol, 1988, 170, 5489–5499; Molec. Plant-Microbe Interact., 1989, 2, 291–300), proved that the substance conforming to the invention is a plant-specific symbiotic signal whose production is under the control of the nod genes.

Indeed:

1) after three types of purifications based on various physicochemical properties, ion-exchange, gel filtration and reversed-phase chromatography, an absolute correlation is observed, in all cases, between the presence of this molecule—characterized by its HPLC profile, by mass spectrometry and by ^{1}H NMR spectrometry—and its specific Had activity on lucerne;

2) an increase in the activity of the nod genes, either by induction of transcription or by increasing the number of copies of these genes, is correlated with an increase in the production of the molecule, whereas a mutation due to the Tn5 transposen in the nodA or nodC genes has the effect of suppressing its production;

3) the biological activity of the compound NodRm-1, as detected by the root hair ramification bioassays, is very high and specific.

A NodRm-1 solution, at a concentration of the order of 10^{-8}–10^{-10}M, causes Had reactions in lucerne, a homologous host, but not in vetch which is a heterologous host. The NodRm-1 compound (see formula II) remains active towards lucerne at a concentration of 10^{-11}M.

The ^{1}H NMR spectrum indicates, for the NodRm-1 compound, a purity of not less than 95% (cf. FIG. 5.1).

Molecules containing an aromatic nucleus, which is characteristic of plant hormones such as auxin and cytokinin, were not detected in the active fraction. Consequently, the possible hormonal contaminants should be present at concentrations of less than 10^{-12}M, that is to say at concentrations about 1000,000 times lower than the threshold (10^{-7}M) at which these phytohormones act. when they are added by an exogenous route, thus indicating that the observed effects cannot be attributed to such contaminants.

The study of the *R. meliloti* mutants affected in the host-specific genes reveals an absolute correlation between the specificity of the symbiotic behavior of the living Rhizobium cells and the specificity of the Had bioassay of their sterile supernatants, thus indicating that the Nod signals are involved in inducing the specific infection and the nodulation.

The NodRm-1 compound has both a very high biological activity and a very high specificity. In fact, it causes reactions which can be detected in the host plant at very low concentrations of the order of 10^{-12}M, whereas known plant hormones such as auxins and cytokinins act at substantially higher concentrations (10^{-7}–10^{-8}M). On the other hand, the "elicitor" type oligosaccharides which have been described in the prior art and which act at low concentrations (10^{-9}M) are not specific, whereas the signal molecule NodRm-1 conforming to the invention has a high specificity of action: while it acts on lucerne, a homologous plant, at a concentration of 10^{-12}M, it remains inactive on a non-host plant such as vetch at a concentration of 10^{-8}M. This molecule therefore exhibits new and important properties with respect to its activity on plants.

In the absence of bacteria and at low concentrations (10^{-7}M–10^{-9}M), the signal molecule NodRm-1 causes the induction of numerous mitotic divisions in the root cortex of lucerne: NodRm-1 therefore exhibits a high mitogenic effect.

In the absence of bacteria and at low concentrations (10^{-7}M–10^{-9}M), the signal molecule NodRm-1 causes the induction of nodosity formation in the lucerne roots, which nodosities exhibit ontological, morphological and anatomical characteristics of the lucerne nodosities induced by symbiotic bacteria. NodRm-1 therefore has an organogenetic effect on the lucerne roots.

In the absence of bacteria and at very low concentrations (10^{-9}M–10^{-12}M), the compound NodRm-1 causes deformations of the root hairs in lucerne and an induction of the transcription of the symbiotic genes of Leguminoseae. At these same doses, in the presence of *R. meliloti*, the addition of the NodRm-1 compound causes an acceleration of the infection and of the formation of root nodosities. Its addition to Leguminoseae, for example by coating the seeds at the time of sowing, is capable of accelerating the formation of nodosities and the establishment of nitrogen fixation.

It is important to underline that it is the first time that oligomers of sulfated hexosamines are isolated from a bacterium. In fact, sulfated sugars have been isolated from animals but were neither detected nor a fortiori isolated up until now either from plants or from bacteria.

Furthermore, the sulfated and acylated hexosamine oligomers conforming to the present invention exhibit a remarkable biological activity in that they possess an activity at infinitesimal doses; indeed, they are at least 100,000 times more active than plant hormones. The use of natural substances having such an activity at such low doses has never been proposed up until now in the plant sector. This remarkable activity suggests that the substances conforming to the invention have a high affinity for certain receptors—an affinity which is substantially higher than that of the products described up until now, including plant hormones. Receptors which have an affinity for sugars generally possess the structure of glycoproteins and in particular lectins; yet such receptors also exist in the animal kingdom; the substances conforming to the invention must therefore be

considered as capable of being endowed with an important therapeutic activity. In particular, the therapeutic agent conforming to the invention is suitable, inter alia, for stimulating the defence mechanisms against pathogens in man and in animals.

Example 3 - Preparation of the substances Ac-NodRm-1 and Ac-NodRM-3 by purification from a *Rhizobium meliloti* culture medium

A. Production of the compounds

If instead of introducing the "overproducing" plasmid pGMI149 into the *R. meliloti* strain 2011, the plasmid pMH682, which contains only the pair of regulatory genes syrM/NodD3, is introduced, a high increase in the transcription of the common and specific nod genes and an increase in the production of Nod factors are also produced. The butanolic extracts of the supernatant of such a strain exhibit, when they are fractionated by HPLC chromatography, a profile different from that of extracts of the *R. meliloti* strain 2011 (pGMI149), thus implying the production of new compounds.

B. Determination of the structure of the compounds

1. Purification

The compounds are purified according to the procedure described in Example 1. The final reversed-phase analytical HPLC chromatography step makes it possible to separate the Ac-NodRMm-1 compound from the Ac-NodRm-3 compound.

2. Structural studies

2.1. Structure of the AC-NodRm-1 compound

2.1.1. Treatment with sodium methanolate

By treating with sodium methanolate, the compound Ac-NodRm-1 is converted to NodRm-1 whose structure is determined as in Example 1.

2.1.2. Mass spectrometry

The positive mode FAB mass spectrum of the compound Ac-NodRm-1 (FIG. **6**A) shows a protonated molecular ion at m/z 1145, as well as the ions $(M+Na)^+$ at m/z 1167 and $(M+2Na-H)^+$ at m/z=1189. Decomposition of the protonated molecular ion by MS/MS spectrometry (FIG. **6**B: B/E scan) leads to fragment ions at m/z 1065, m/z 844, m/z 641 and m/z 438. The fragment ions at m/z 1065 and m/z 884 are attributed to the loss of SO_3 and N-acetyl-D-glucosamine sulfate respectively. The fragments at m/z 641 and m/z 438 correspond to the glycosylium ions obtained by breaking the interglycoside bonds. These two spectra show that the compound Ac-NodRm-1 differs from the compound NodRm-1 only by the presence of an acetate group on the nonreducing terminal glucosamine.

2.1.3. ^1H NMR

The ^1H NMR spectrum of the compound Ac-NodRm-1 makes it possible to establish the structural elements described in Example 1 (paragraph B.4.1.) for the compound NodRm-1.

(i) two double bonds including one which is conjugated, of E configuration;

(ii) anomeric signals characteristic of β bonding

(iii) signals characteristic of a sulfate group in position 6.

Furthermore, two signals at δ=4.16 ppm and δ=4.46 ppm, which are absent from the spectrum of the compound NodRm-1 and disappearing after O-deacetylation, make it possible to localize the acetate group in position 6 of the nonreducing terminal sugar.

2.2. Structure of the compound Ac-NodRm-3

The structure of the compound Ac-NodRm-3 was established by positive mode FAB mass spectrometry (FIG. 7). A protonated molecular ion is observed at m/z=1348 as well as sodium-containing ions $(M+Na)^+$ at m/z=1370 and $(M+2Na-H)^+$ at m/z=1392. The ions at m/z=1268 and m/z=1047 are respectively attributed to the loss of SO_3 and an N-acetylglucosamine sulfate from $(M+H)^+$. The ions m/z=844, m/z=641 and m/z=438 are attributed to the glycosylium ions obtained by interglycoside cleavages. This spectrum indicates that Ac-NodRm-3:

(i) consists of a linear chain of five D-glucosamine derivatives;

(ii) possesses a diunsaturated N-acyl chain of 16 carbons and an O-acetate group on the nonreducing terminal glucosamine residue;

(iii) possesses a sulfate functional group on the reducing N-acetyl-D-glucosamine residue.

Incubation of the Ac-NodRm-3 compound in the presence of an exochitinase (extracted from *Streptococcus griseus*, Sigma) releases in particular an N-acetyl-D-glucosamine and N-acyl-O-acetyl-D-glucosamine dimer, which shows that the bonds between the first three N-acetyl-D-glucosamine residues are of the β-1,4 type. Digestion of Ac-NodRm-3 by an endochitinase (also extracted from *Streptococcus griseus*, Boeringer) leads to the formation of an N-acyl-O-acetyl-D-glucosamine showing that the bonding with the nonreducing terminal residue is also of the β-1,4 type.

Example 4 - Preparation of Nod factors from a *Rhizobium leguminosarum* biovar viciae culture medium

A. Production of the Nod factors

In European countries, the cultivation of protein-rich plants such as pea and field bean developed considerably during the last decade. The inventors therefore set out to study the production of the Nod factor by *Rhizobium leguminosarum* b.v. *viciae*, the symbiotic bacteria for these protein-rich plants.

The inventors introduced the plasmid plJ1089 into the *R. leguminosarum* strain 248 with the aim of causing an increase in the production of these signals. This plasmid, which is derived from the vector pLAFR1, contains the entire Nod region of *R. leguminosarum*, that is to say the operons nodD, nodABCIJ, nodFEL, nodMNT, nodO (Downie et al., EMBO J., 1983, 2, 947–952).

Strain 248 (plJ1089) is cultured in the same medium as *R. meliloti* but for inducing the transcription of the nod genes, a flavanone, naringenin, was used in place of luteolin. After inducing the nod genes, the culture supernatant exhibits a high activity of deformation of the root hairs of a suitable vetch species (*Vicia sativa* subsp. *nigra*). This activity is not detectible in the culture supernatants of an *R. leguminosarum* strain 248 which is mutated in the common genes nodABC.

B. Purification

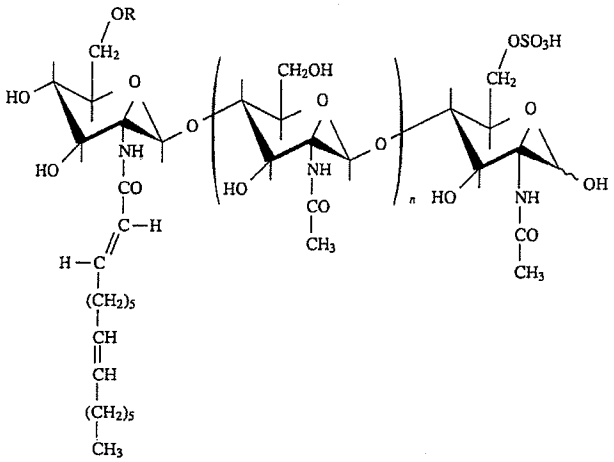
The Nod compounds are extracted from the culture medium according to the procedure described in Example 1 (B.1.). The extract is freeze-dried and then chromatographed by reversed-phase HPLC according to the procedure described in Example 1 (B.1.2.). The Nod compounds are then purified on a Sep-Pak QMA cartridge (Waters Millipore) in the acetate form. The compounds are eluted with 5 ml of absolute ethanol.

C. Determination of the structure

The positive mode FABmass spectrum predominantly shows three protonated molecular ions at m/z=1067, m/z=1069 and m/z=1095, accompanied by their sodium-containing ions $(M+Na)^+$ at m/z=1089, m/z=1091 and m/z 1117

respectively. Decomposition, by MS/MS spectrometry, of the ion at m/z=1089 (m/z=1091 and m/z=1117 respectively) provides the fragment ions at m/z=868 (m/z=870 and m/z=896 respectively), m/z=665 (m/z=667 and m/z=693 respectively) and m/z=462 (m/z=464 and m/z=490 respectively). These ions are obtained by interglycoside rupture, with the successive loss of fragments α with a mass of 203, corresponding to an N-acetylglucosamine residue. These spectra show that the NodR1 compounds consist of a linear N-acetylhexosamine chain. The ions of m/z=462, 464 and 490 correspond to variously N-acylated O-acetylhexosamines.

Analysis of the fatty acids carried by the nonreducing terminal residue was carried out by GC/MS after saponification according to the procedure described in paragraph B.

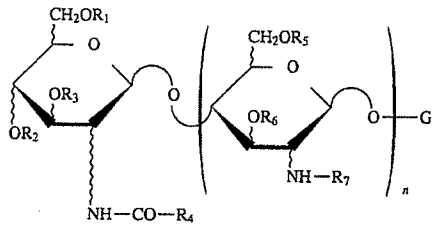


2.2. This study made it possible to identify the following predominant fatty acids: $C_{16:0}$, $C_{15:1}$ and $C_{18:1}$.

As evident from the above, the invention is not in the least limited to the implementations, embodiments and applications which have just been described more explicitly; on the contrary, it embraces all the variants which may come to the mind of a specialist in this field without departing from the framework or the scope of the present invention.

We claim:

1. A purified lipo-oligosaccharide having formula I below:



wherein G is selected from the group consisting of hexosamine, acetyl-substituted hexosamine, sulphated-substituted hexosamine, and ether-substituted hexosamine,

R_1, R_2, R_3, R_5, R_6 and R_7, which are identical or different, are selected from the group consisting of H, C_xH_yCO—, and carbamyl, wherein x is an integer between 0 and 17, and y is an integer between 1 and 35,

R_4 is selected from the group consisting of a mono-, di-, or tri-unsaturated aliphatic chain containing at least 12 carbon atoms, and

n is an integer between 1 and 4.

2. The lipo-oligosaccharide of claim **1**, wherein G represents a compound selected from the group consisting of N-acetyl-D-glucosamine 6-sulphate and N-acetyl-D-glucosamine.

3. The lipo-oligosaccharide of claim **1**, having formula (II) below:

in which R is H or CH_3CO— and n is equal to 2 or 3.

4. A composition for treating plants, comprising the lipo-oligosaccharide of claim **1**, and an agriculturally suitable carrier.

5. The composition of claim **4**, wherein the concentration of lipo-oligosaccharide is between 10^{-6}M and 10^{-14}M.

6. A method for enhancing the capacity of a bacteria of the family Rhizobiacae to infect a host plant of the family Leguminoseae comprising administration to said plant of an amount effective to act as plant-specific symbiotic signal, of a lipo-oligosaccharide of claim **1**.

7. The method of claim **6**, wherein said lipo-oligosaccharide is at a concentration between 10^{-6}M and 10^{-14}M.

8. A method of accelerating the formation of nodules on a host plant of the family Leguminoseae comprising administration to said plant of an amount effective to act as plant-specific symbiotic signal, of a lipo-oligosaccharide of claim **1**.

9. The method of claim **8** wherein said lipo-oligosaccharide is at a concentration between 10^{-6}M and 10^{-14}M.

10. A method of inducing the transcription of the symbiotic genes of Leguminoseae comprising administration to a Leguminoseae of an amount effective to act as plant-specific symbiotic signal, of a lipo-oligosaccharide of claim **1**.

11. The method of claim **10** wherein said lipo-oligosaccharide is at a concentration between 10^{-6}M and 10^{-14}M.

* * * * *

EXHIBIT C

US006979664B1

(12) **United States Patent**
Smith et al.

(10) **Patent No.: US 6,979,664 B1**
(45) **Date of Patent: Dec. 27, 2005**

(54) **COMPOSITION FOR ACCELERATING SEED GERMINATION AND PLANT GROWTH**

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(52) **U.S. Cl.** **504/117**; 504/292

(58) **Field of Search** 504/117, 113, 313, 504/292

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(Continued)

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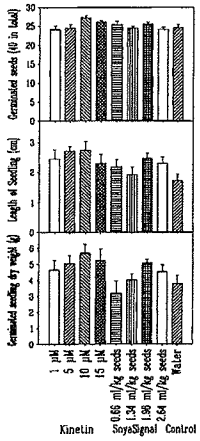
(57) **ABSTRACT**

Lipo Chitooligosaccharide (LCO) [NodBj-V(C18:1,Me-fuc)] isolated from *Bradyrhizobium japonicum* strain 532C was able to stimulate seed germination/seedling emergence, or in the case of potato, sprouting, of a number of crop plants representing eight distantly related plant families (Poaceae, Fabaceae, Brassicaceae, Cucurbitaceae, Malvaceae, Asteraceae, Chenopodiaceae and Solanaceae) of plants, at 25 and/or at 15° C. It also promoted sprouting potato minitubers. Other LCOs [NodRM-V($C_{16:2,5}$) and LCO from *R. leguminosarum*] were also shown to also display growth-promoting effects on the tested crop plants. The compositions comprising at least one LCO are shown to be effective in promoting growth under both laboratory and field conditions. The invention thus also relates to methods for promoting seed germination and/or seedling emergence and/or growth of plants comprising subjecting the seeds and/or plants to an effective amount of an agricultural composition comprising at least one LCO.

35 Claims, 1 Drawing Sheet



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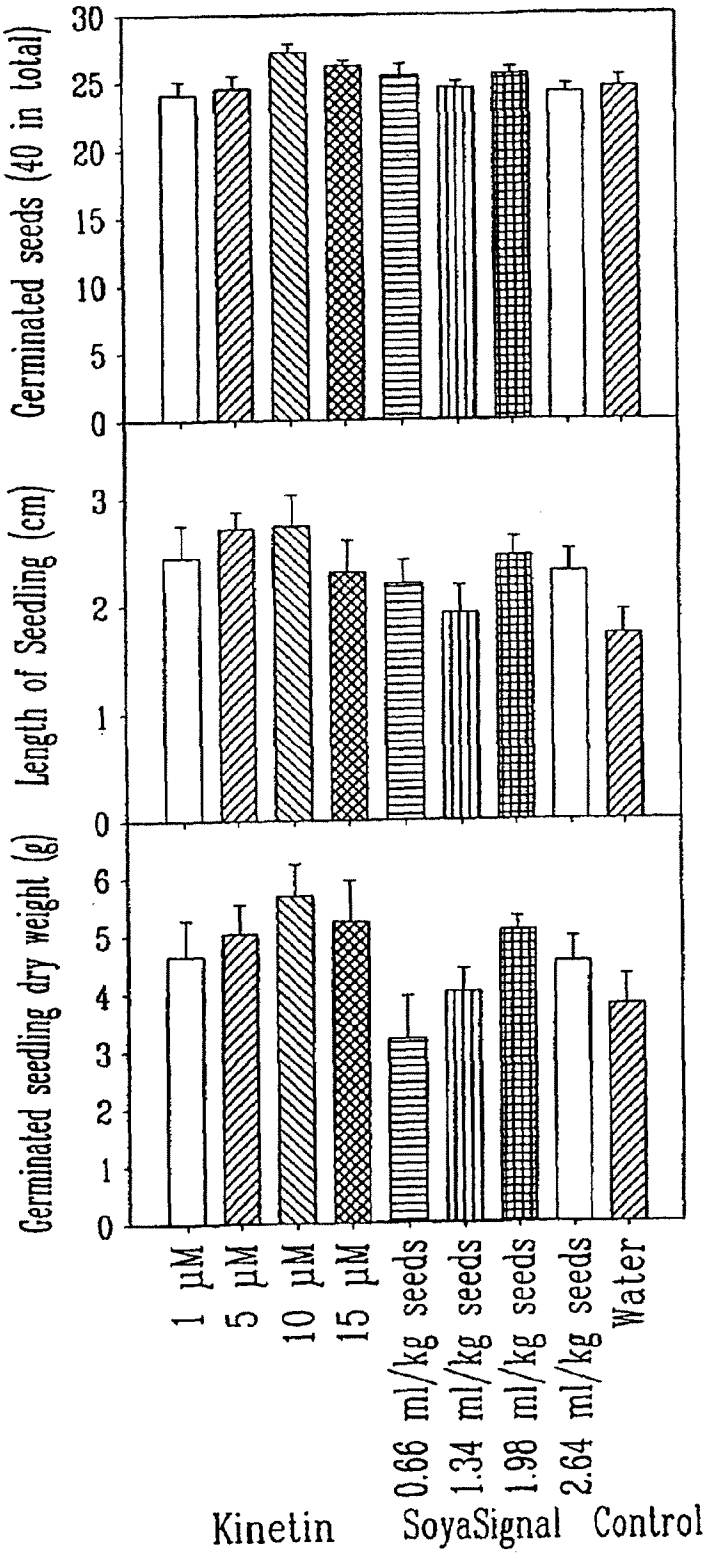

Germinated seeds (40 in total)
30
25
20
15
10
5
0
Length of Seedling (cm)
3
2
1
0
Germinated seedling dry weight (g)
6
5
4
3
2
1
0
1 µM
5 µM
10 µM
15 µM
0.66 ml/kg seeds
1.34 ml/kg seeds
1.98 ml/kg seeds
2.64 ml/kg seeds
Water
Kinetin
SoyaSignal
Control

COMPOSITION FOR ACCELERATING SEED GERMINATION AND PLANT GROWTH

FIELD OF THE INVENTION

The present invention relates to agriculture. More specifically, the invention relates to plant seed germination, seedling emergence, quiescence-breakage and plant growth. Even more specifically, the present invention relates to compositions which accelerate plant seed germination, seedling emergence and plant growth of numerous types of crops and to methods using same.

BACKGROUND OF THE INVENTION

Symbiotic microorganisms can promote the growth of legumes by way of biological fixation of nitrogen. More specifically, rhizobiaceae are gram-negative soil bacteria which fix nitrogen and are involved in symbiotic association with these legumes. This symbiotic association between the bacteria and the legume enables the latter to grow in soils having low assimilable nitrogen levels. In return, through photosynthesis, the legume provides the bacteria with the energy it requires to reduce the atmospheric nitrogen into ammonia. This ammonia can then be used by the legume and enters into the nitrogen metabolism. The legume, of the Fabaceae family, forms nodules in which the rhizobia proliferate. The Rhizobiaceae family is in a state of taxonomic flux. It has been reported to comprise four main genera: *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Azorhizobium* (U.S. Pat. No. 5,549,718). The symbiotic relationship between nitrogen-fixing bacteria or rhizobia and plants of the Fabaceae family enables the growth of the latter in soils having low levels of available nitrogen, thus reducing the need for nitrogen fertilizers. Since nitrogen fertilizers can significantly increase the cost of crops, and are associated with a number of polluting effects, biological means to stimulate this symbiotic relationship and/or to decrease the use of nitrogen fertilizers is of great importance.

Initial recognition between *B. japonicum* and soybean involves exchange of molecular signals (Stacey et al, 1995). Legume roots secrete phenolic compounds (Dakora & Philips, 1996; Peters & Verma, 1990), largely from the area of root hair emergence, which act as chemo-attractants to (brady)rhizobia (Nap & Bisseling, 1990), and activate the nod genes. Flavones, isoflavones, and flavanones have been identified as the inducing molecules for (brady)rhizobial chemotaxis and for expression of nod genes, e.g. genistein, daidzein and several related compounds in soybean (Peters & Verma, 1990). These plant-to-bacteria signal compounds cause expression of the bacterial nod (also nol and noe) genes very rapidly (only a few minutes after exposure) and at very low concentrations (10^{-7} to 10^{-8} M) (Peters et al., 1986). Generally this is through an interaction with nodD, which activates the common nod genes, although the situation may be more complex, as is the case in *B. japonicum*, where $nodD_1$, $nodD_2$ and nodVW are involved (Gillette & Elkan 1996; Stacey 1995). Nod genes have been identified in the rhizobia that form nitrogen fixing relationships with numbers of the Fabiaceae family (see U.S. Pat. No. 5,549, 718 and references therein). Recently, the plant-to-bacteria signal molecules have been shown to promote soybean nodulation and nitrogen fixation under cool soil temperatures (CA 2,179,879) and increase the final soybean grain yield on average of 10% in the field and up to 40% under certain conditions.

Among the products of the nod genes induced by the plant phenolic signal molecules are various enzymes involved in the synthesis of a series of lipo chitooligosaccharides (LCOs) (Spaink, 1995; Stacey, 1995). These newly synthesized LCOs act as bacterium-to-plant signals, inducing expression of many of the early nodulin genes (Long, 1989). This results in root hair deformation (including curling), cortical cell division leading to initiation of nodule meristems, secretion of additional nod gene inducers, and initiation of infection threads (Verma, 1992). These bacterium-to-plant signals exert a powerful influence over the plant genome and, when added in the absence of the bacteria, can induce the formation of root nodules (Truchet et al., 1991). Thus, the bacteria-to-plant signals can, without the bacteria, induce all the gene activity for nodule organogenesis (Denarie et al., 1996; Heidstra & Bisseling, 1996). Moreover, the above-mentioned activities induced by LCOs can be produced by concentrations as low as 10^{-14} M (Stokkermans et al. 1995). The mutual exchange of signals between the bacteria and the plant are essential for the symbiotic interaction. *Rhizobia* mutants unable to synthesize LCOs will not form nodules. Analysis of the *B. japonicum* nod genes indicates that ability to induce soybean nodulation requires at least: 1) a basic tetrameric Nod factor requiring only nodABC genes or 2) a pentameric LCO(C18:1, C16:0 or C16: fatty acid and a methyl-fucose at the reducing end, sometimes acetylated) requiring nodABCZ genes (Stokkermans et al. 1995).

When added to the appropriate legume, LCOs can cause the induction of nodule meristems (Denarie et al., 1996), and therefore cell division activity. One previous publication has shown that LCOs can induce cell cycle activities in a carrot embryogenesis system at levels as low as 10^{-14} M (De Jong et al. 1993).

A chemical structure of lipo chitooligosaccharides, also termed "symbiotic Nod signals" or "Nod factor", has been described in U.S. Pat. Nos. 5,549,718 and 5,175,149. These Nod factors have the properties of a lectin ligand or lipo-oligosaccharide substances which can be purified from bacteria or synthesized or produced by genetic engineering.

The relationship between environmental variables, such as low root zone temperature (RZT) and pH, and the interplay of molecular signals has only recently become a subject of investigation. For example, some soybean genotypes have less synthesis abilities for isoflavones under cool soil temperature, whereas a higher isoflavone concentration is needed to turn on the nod genes of *B. japonicum* (Zhang and Smith 1995 and 1997). The plant-to-bacteria signal molecules (i.e. isoflavones) have been shown, among other things, to overcome the negative effect of low temperature on the early events of symbiotic nitrogen fixation (Canadian application number 2,179,879).

While the effects of plant-to-bacteria signal molecules (i.e. isoflavones) on nodulation, nitrogen fixation, growth and protein yield of legumes, such as soybean, and on bacteria-to-plant signal molecules (LCOs) on nodulation and nitrogen fixation in legumes have been described under certain conditions, the effect of the bacteria-to-plant signal molecules on the growth of non-legumes is unknown. In fact, the role of such bacteria-to-plant signal molecules on non-legumes has never been assessed. In addition, the effect of LCOs on processes other than nodulation of legumes has yet to be studied.

There thus remains a need to assess the effect of LCOs on seed germination, seedling emergence and/or growth of plants in general and especially of non-legume plants.

The present invention seeks to meet these and other needs.

The present description refers to a number of documents, the contents of which are herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The invention concerns a composition for enhancing seed germination, seedling emergence and growth of plants and especially of crop plants. More specifically, the present invention relates to a composition comprising an LCO which can increase seed germination and/or seedling emergence and/or growth of a legume, in addition to acting as a trigger to initiate legume symbiotic nitrogen fixation. More particularly, the invention relates to increased seed germination and/or seedling emergence and/or growth of soybean, pea and red clover.

Surprisingly, the compositions of the present invention act not only on legumes such as soybean but on plants in general as exemplified with non-legume crops from different plant families Poaceae, Cucurbitaceae, Malvaceae. Asteraceae, Chenopodiaceae and Solonaceae. More specifically, the non-legume crops exemplified herein include corn, cotton, cantaloupe, cucumber, canola, lettuce, potato and beet. The present invention thus also refers to compositions for enhancing seed germination and/or seedling emergence and/or growth of non-legumes. More particularly, the invention relates to compositions comprising an LCO for enhancing seed germination, seedling emergence and growth of non-legumes. Non-limiting examples of such non-legumes include cotton, corn, canola, potato, cucumber, cantaloupe, lettuce and beet. Broadly, the present invention relates to compositions comprising an LCO for promoting growth of a crop. Non-limiting examples of crop plants include monocot, dicot, members of the grass family (containing the cereals), and legumes.

Thus, the present invention relates to agricultural compositions comprising at least one LCO (and methods of using same) for promoting seed germination, and/or early development of seedlings, and/or emergence of sprouts from tubers, and/or rapid development of new plants from higher plant perinating structures.

In a particular set of experiments in the field, a composition of the present invention comprising an LCO was shown to significantly enhance early plant growth.

The invention in addition relates to methods for enhancing seed germination and/or seedling emergence and/or growth of plants and/or for breaking the dormancy thereof comprising a treatment in the vicinity of a seed or seedling or plant with an effective amount of an agricultural composition comprising an LCO and an agriculturally suitable carrier for a sufficient time and under conditions which enable an increased germination of the seed and/or an increased emergence of the seedling and/or an increased growth of the plant and/or a triggering of the growth of a dormant plant.

The invention also relates to compositions and methods for breaking the dormancy of a plant and initiating the growth thereof. In a particular embodiment, the invention relates to the breaking of dormancy of potato.

The Applicant is the first to show that a composition comprising an LCO can have a significant effect on seed germination, and/or seedling emergence of legumes. Moreover, the Applicant is the first to show the surprising effect of signal molecules involved in bacteria-legume signalling on the growth of non-legume plants. In addition, the Applicant is the first to show that a composition comprising an LCO had an effect on non-legume seed germination and/or seedling emergence and/or plant growth of the non-legume. Also, the Applicant is the first to show that an LCO can not only act as a dormancy breaker but that it can also significantly increase the yield of a dormant plant following the dormancy breakage, when compared to known dormancy breakers.

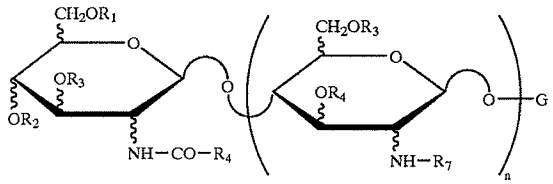
While the seed germination and/or seedling emergence and/or plant growth enhancing capabilities of the compositions of the instant invention are demonstrated with corn, cotton, canola, potato, cantaloupe, lettuce, beet, cucumber, soybean, pea and red clover, they are applicable to plants in general and more especially to crop plants. Indeed, the plants chosen for the experiments presented herein are crops from significantly divergent plants in eight distinct families: (1) corn, the only monocot tested herein, in the family of grasses (Poaceae), which also contains the cereals; (2) cucumber and cantaloupe, the latter being a plant used horticulturally, and being slow to germinate at low temperature [its base temperature is about 14° C.] (Cucurbitaceae); (3) cotton, one of the most important fibre crops on the planet (Malvaceae); (4) lettuce (Asteraceae); (5) beet (Chenopodiaceae); (6) potato, a very important crop (Solonacea, which also includes tobacco, peppers and tomato); and two families of legumes (7) canola, representing the mustard group (Brassicaceae) and (8) soybean (representative of oil seed crop), bean (representative of a crop for human consumption) and red clover and alfalfa (forage legumes) (all of the Fabaceae family).

In view of the diversity of the plants tested, and of the similar results obtained with these different crop plants, it can be predicted that such results will apply to crop plants in general. It follows that a person skilled in the art can adapt the teachings of the present invention to other crops. Non-limiting examples thereof include tobacco, tomato, wheat, barley, rice, sunflower and plants grown for flower production (daisy, carnation, pansy, gladiola, lilies and the like). It will be understood that the compositions can be adapted to specific crops, to meet particular needs.

In accordance with the present invention, there is thus provided an agricultural composition for enhancing plant crop seed germination and/or seedling emergence and/or growth of a plant crop comprising a growth-promoting amount of at least one lipo chitooligosaccharide (LCO) together with an agriculturally suitable carrier. There is also provided a composition for breaking the dormancy and/or quiescence of a plant, comprising a growth-promoting amount of at least one lipo chitooligosaccharide (LCO) together with an agriculturally suitable carrier. Furthermore, there is provided a method for enhancing seed germination and/or seedling emergence and/or growth of a plant, comprising a treatment in the vicinity of one of a seed, root or plant with a composition comprising an agriculturally effective amount of a lipo chitooligosaccharide (LCO) in admixture with an agriculturally suitable carrier medium, wherein the effective amount enhances seed germination and/or seedling emergence and/or growth of the plant in comparison to an untreated plant. There is further provided a method for enhancing seed germination and/or seedling emergence and/or growth of a plant crop comprising incubating a rhizobial strain which expresses a lipo chitooligosaccharide (LCO) in the vicinity of one of a seed and/or root of the plant such that the LCO enhances seed germination and/or seedling emergence and/or growth of the plant crop.

As used herein, the term "rhizobia" is used broadly to refer to bacterial strains which are involved in a nitrogen fixing symbiotic relationship with a legume.

As used herein, the term "LCO" refers broadly to a Nod factor which is under the control of at least one nodulation gene (nod gene), common to rhizobia. LCO therefore relates to a bacteria-to-plant signal molecule which induces the formation of nodules in legumes and enables the symbiotic bacteria to colonize same. Broadly, LCOs are lipo chitooligosaccharide signal molecules, acting as phytohormones, comprising an oligosaccharide moiety having a fatty acid condensed at one of its end. An example of an LCO is presented below as formula I



in which:

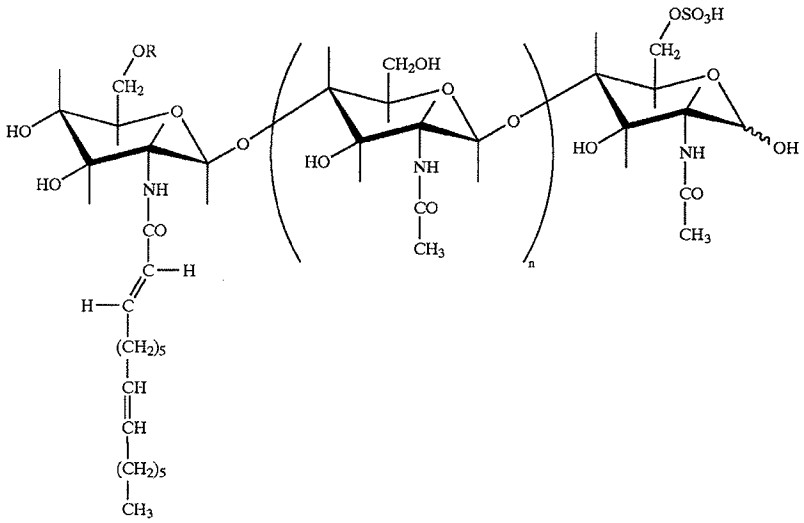
G is a hexosamine which can be substituted, for example, by an acetyl group on the nitrogen, a sulfate group, an acetyl group and/or an ether group on an oxygen,

R_1, R_2, R_3, R_5, R_6 and R_7, which may be identical or different, represent H, CH_3CO—, C_xH_yCO— where x is an integer between 0 and 17, and y is an integer between 1 and 35, or any other acyl group such as for example a carbamyl,

R_4 represents a mono-, di- or triunsaturated aliphatic chain containing at least 12 carbon atoms, and

n is an integer between 1 and 4.

More specific LCOs from *R. meliloti* have also been described in U.S. Pat. No. 5,549,718 as having the formula II



in which R represents H or CH_3CO— and n is equal to 2 or 3.

Even more specific LCOs include NodRM, NodRM-1, NodRM-3. When acetylated (the R═CH_3CO—), they become AcNodRM-1, and AcNodRM-3, respectively (U.S. Pat. No. 5,545,718).

LCOs from *B. japonicum* have also been characterized in U.S. Pat. Nos. 5,175,149 and 5,321,011. Broadly, they are pentasaccharide phytohormones comprising methylfucose. A number of these *B. japonicum*-derived LCOs are described: BjNod-V ($C_{18:1}$); BjNod-V (A_C, $C_{18:1}$), BjNod-V ($C_{16:1}$); and BjNod-V (A_C, $C_{16:0}$), with "V" indicating the presence of five N-acetylglucosamines; "Ac" an acetylation; the number following the "C" indicating the number of carbons in the fatty acid side chain; and the number following the ":" the number of double bonds.

It shall also be understood that compositions comprising different LCOs, are encompassed within the scope of the present invention. Indeed, while the present invention is exemplified with NodBj-V($C_{18:1\Delta}$)11 also known as BjNod-V($C_{18:1}$MeFuc); NodRM-V($C_{16:2}$, S); and NodRI, any LCO produced by a rhizobia which is capable of entering into a nitrogen fixation relationship with a legume (i.e. a member of the Fabiaceae family) is expected to have the potential to show the same properties as those described herein. It will be clear to the person of ordinary skill that the selection of a rhizobia known to be expressing LCOs at high levels, or known to express an LCO having an effect on a broader spectrum of legumes could be advantageous.

It will also be clear that the LCO compositions of the present invention could also comprise more than one signal molecule. Non-limiting examples of such compositions include agricultural compositions comprising in addition to one LCO: (1) at least one additional LCO; (2) at least one plant-to-bacteria signal molecule; (3) gibberellic acid or other agents or compounds known to promote growth or fitness of plants; and mixtures of such compositions (1), (2) or (3).

It shall be clear that having identified new uses for LCO, bacteria could be genetically engineered to express nod genes and used for producing LCOs or for direct administration to the plants and/or seeds.

Thus, while the instant invention is demonstrated in particular with LCOs from *Bradyrhizobium* japonicum, *Rhizobium* meliloti and *R. leguminosarum* and selected legumes and non-legume crops, the invention is not so limited. Other legume crops, non-legume crops and rhizobial strains may be used using the same principles taught herein. Preferred matching of rhizobia with legume crop groups include, for example:

rhizobial species	Legume crop group
R. meliloti	alfalfa, sweet clover
R. leguminosarum	peas, lentils
R. phaesolii	beans
Bradyrhizobium japonicum	soybeans
R. trifolii	red clover

As will be apparent to the person of ordinary skill to which the present invention is directed, the growth-stimulating compositions of the present invention can be applied to other crop plants and especially to other warm climate adapted crop plants (plants or crops having evolved under warm conditions [i.e. tropical, subtropical or warm temperature zones] and whose metabolism is optimized for such climates). It should be understood that the growth-enhancing compositions of the present invention should find utility whenever a particular crop is grown in a condition which limits its growth. More particularly, whenever a particular plant crop is grown at a temperature which is below its optimum temperature for seed germination, seedling emergence, growth and the like. Such temperatures are known in the art. For example, optimum temperatures for germination of corn, soybean, rice and cotton are 30° C., 34–36° C., 30–32° C., and 34° C., respectively. The minimum germination temperatures (or base temperatures) for these crops are 9° C., 4° C., 8 to 10° C., and 14° C., respectively, while the maximum germination temperatures are 40° C., 42–44° C., 44° C. and 37° C., respectively. The compositions of the present invention therefore find utility, among other things, in enhancing germination of warm climate adapted crops when grown at temperatures between their base temperature for seed germination, and/or seedling emergence and/or growth and their optimum temperature for germination. The compositions of the present invention find utility in general in enhancing seed germination and/or se dling emergence and/or growth of crop plants when grown under conditions which delay or inhibit seed germination and/or seedling emergence thereof. Non-limiting examples of such inhibiting conditions (as known from their signalling inhibition in bacteria-legume interactions, their inhibition or delay of the bacteria-plant symbiotic relationship) include pH stress, heat-stress, and water stress.

It will be nevertheless recognized that the compositions and methods of the present invention enhance growth of plants grown under optimal conditions.

Thus, the compositions and methods of the present invention should not be limited to plants growing under sub-optimal conditions.

The term "environmental conditions which inhibit or delay the bacterial-plant symbiotic relationship" should be interpreted herein as designating environmental conditions which postpone or inhibit the production and exchange of signal molecules between same and include, without being limited thereto: conditions that stress the plant, such as temperature stress, water stress, pH stress as well as inhibitory soil nitrogen concentrations or fixed nitrogen.

"An agriculturally effective amount of a composition" for increasing the growth of crop plants in accordance with the present invention refers to a quantity which is sufficient to result in a statistically significant enhancement of growth and/or of protein yield and/or of grain yield of the plant crop as compared to the growth, protein yield and grain yield of the control-treated plant crop. As will be seen below, the growth promoting activity of the LCOs are observable over a broad range of concentrations. Indeed, LCO growth-promoting activities can be observed at an applied concentration of about 10^{-5} to 10^{-14} M, preferably about 10^{-6} to about 10^{-12} M and more preferably about 10^{-7} to about 10^{-10}M.

The term "immediate vicinity of a seed or roots" refers to any location of a seed or roots wherein if any soluble material or composition is so placed, any exhibit of the plant or of the bacteria, or bacterial cells will be in actual contact with the seed as it germinates or the roots as they grow and develop.

Direct or indirect methods of inoculation with the composition of the present invention can be employed. During direct inoculation the composition is applied directly to the seed prior to sowing. This can most simply be accomplished by spraying the seed with or dipping the seed into a liquid culture containing the desired components.

The recitation "short season condition" refers herein broadly to temperatures of the middle and temperate zones and shorter. Typically, the active growing season is around ½ to ⅔ of the year. Short season conditions broadly refers to a frost-free period of less than half the year, often on the order of 100 frost-free days.

By "nodulation gene-inducing" or "nod gene-inducing" is meant bacterial genes involved in nodule establishment and function.

By "seed germination" is meant a clear evidence of root growth developing from the embryo on the seed. When referring to an "increased seed germination", the Applicant refers to a significant difference in seed germination between the treated versus the control seed.

"Seedling emergence" is meant to refer to growth of the plant which is observable above the rooting medium surface. When referring to an "enhanced seedling emergence", the Applicant refers to a significant observable difference between the growth of the seedling in the treated versus the control.

BRIEF DESCRIPTION OF THE DRAWING

Having thus generally described the invention, reference will now be made to the accompanying drawing, showing by way of illustration a preferred embodiment thereof, and in which:

FIG. **1** shows the seed germination enhancing effect of a composition according to the present invention on corn.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

While the effects on nodulation were detected upon treatment of soybean with SoyaSignal® (a composition comprising both the plant-to-bacteria and bacteria-to-plant

signal molecules), it was also noted that in many of the field experiments the plants that received some sort of genistein treatment emerged from the soil sooner. Thus, an experiment, in which genistein alone, *B. japonicum* alone, and genistein plus *B. japonicum* were added to soybean seeds under controlled environment conditions, was conducted. Because slow germination of corn (and other plants, as well) is a serious agricultural problem in eastern Canada because the weather conditions limit the growth thereof, corn was also treated in a similar fashion. The experiment showed that the seed germination and seedling emergence promoting effect was present with the combination of genistein plus *B. japonicum*, leading to the conclusion that the enhancing effects were due to the LCOs produced by genistein exposed *B. japonicum*. Purification (HPLC and otherwise) of the LCO most abundantly produced by genistein-stimulated *B. japonicum* (NodBj-V($C_{18:1\Delta}$11)) was carried out. This was aided by the gracious gift of enough LCO material to standardize the assay (G. Stacey, University of Tennessee at Knoxville; U.S. Pat. No. 5,175,149 and U.S. Pat. No. 5,321,011) which allowed both isolation and quantification. With isolated NodBj-V($C_{18:1\Delta}$11), research on the ability of this compound to stimulate seed germination, seedling emergence and growth of leguminous and non-leguminous plants could be conducted.

These experiments surprisingly demonstrated that the addition of SoyaSignal (which comprises both an isoflavone and an LCO; the latter at a concentration of about 105M) accelerates the germination of corn seeds, whereas isoflavone solutions alone do not. Presumably this effect was due to the LCOs produced by *B. japonicum* cells and induced by the presence of isoflavones. When the seedlings were harvested (still at the mesocotyl stage) they were 44% longer and 33% as heavier in the genistein-*B. japonicum* treated versus non-treated plants (FIG. **1**). In addition, not only did seedling emergence increase, but the rate of cotton seed germination was also accelerated by the application of SoyaSignal®. The germination rate of cotton seeds treated with SoyaSignal® (0.66 ml/kg seed) increased by 145% compared to those control seeds that were treated with pure water. Both the corn and cotton experiments were conducted at low temperatures, 15° C. and 17° C. for corn seeds and for cotton seeds, respectively.

The field trial showed that the time of tasselling of sweet corn treated with SoyaSignal® (planted on May 6 on the Experimental Farm of McGill University, Quebec) was 1 to 2 days earlier compared to that of untreated plants. Soybean seeds that received SoyaSignal® (planted on June 22 in Martinsville, Ill.) emerged 8 hours earlier compared to control seeds while the first trifoliar fully expanded 1 day earlier. At the agronomy farm of Purdue University, Ind., soybeans planted in early June and observed in early July were already one stage further in their development (V6) compared to the control plants (V5). In a farmer trial (in Jackson, Ill.), plants that received SoyaSignal® had many more nodules on the secondary roots and were 10% taller than untreated plants.

Thus, an LCO (a bacteria-to-plant signal molecule involved in the establishment of the symbiotic relationship between a rhizobia and a legume) can promote growth of corn, a monocot distantly related to legumes. Based on the evolutionary divergence of corn from legumes and the significant response thereof to the LCO treatments, corn was used as a model plant system in follow-up experiments. These experiments demonstrated that the results obtained with corn were also observable with all other crop plants tested.

Taken together, the laboratory data and field trials presented herein show that an LCO can increase seed germination, seedling emergence and plant growth of legumes and non-legume plants under controlled environment and field conditions.

The signal molecules are also shown to break the dormancy of potato tubers. Of note, the dormancy experiments showed that the signal solution was better at increasing the yield of potato tubers as compared to other dormancy breakers (i.e. giberellic acid).

The precise mechanism of action of LCOs on seed germination, seedling emergence dormancy and plant growth of legumes and non-legumes is not fully understood. The general understanding of the role of LCOs in signalling during the establishment of the legume-rhizobia symbiosis was described above. When added to the appropriate legume, LCOs can cause the induction of nodule meristems. Thus, it is possible that LCOs might be normal signal molecules in higher plants, so that exogenously supplying them simply increases their levels and, therefore, the activity of the things they would normally regulate. Alternatively, there may be an endogenous class of signal molecules which play important roles in plant development, and have a conformation similar to those of LCOs. One possible candidate for this is the oligosaccharins (Fry et al. 1993), some of which do stimulate meristem activity (Pavlova et al. 1992). LCOs are somewhat similar in structure and chemistry to the oligosaccharins (Fry et al. 1993) and can, in the broadest sense, be included in that group (Stokkermans et al. 1995). However, the signal molecules with a similar conformation need not be chemically similar, as demonstrated by the ability of opiates (plant alkaloids) to fit into receptor sites normally occupied by endorphrins (oligo-peptides). Nothing is known regarding the mechanisms by which LCOs cause this activity. Without being limited to a particular theory, the present invention is nevertheless the first to have identified a seed germination, and/or seedling emergence and/or dormancy breaking and/or plant growth promoting effect of a composition comprising LCOs on non-legume plants.

Crops, such as soybean, corn and cotton evolved in relatively warm climates and, as a result, have high base temperatures for germination, being of about 5° C. for soybean, 10° C. for corn and 14° C. for cotton. These high base germination temperatures lead to slow emergence after planting, resulting in slow leaf ground cover early in the season (when the temperature is sub-optimal), which in turn leads to poorer early season light interception, poorer competition with weeds (and therefore greater need for herbicide application) and increased soil erosion during heavy rainfall events. To simplify, these crops are often grown under conditions which limit their seed germination, and/or seedling emergence and/or growth. Hence, the use of a growth-promoting factor which is in limiting amount can compensate for a deficiency or stress in the growth conditions. Using SoyaSignal® as a plant growth regulator could thus partially overcome the negative effects of environmental stress conditions, such as low soil temperature on crop seed germination, seedling emergence and plant development. Thus, the present invention provides the means to improve the production of crops of tropical and subtropical origin in the temperate zones and may extend their production into shorter season areas. In addition, the present invention provides the means to improve production of crops growing under stress conditions.

The present invention is illustrated in further detail by the following non-limiting examples.

EXAMPLE 1

Induction of LCO Production by *Bradyrhizobium japonicum*

The first culture containing *Bradyrhizobium japonicum* (strain 532C) was grown at 28° C. in 100–125 mL of sterile yeast mannitol media (YEM) with pH 6.8, shaken at 150 rpm until the OD_{620} reaches 0.4–0.6 (4–6 days). Thereafter, a 2L bacterial subculture was started by inoculating with material from the first culture (5 mL of first culture per 250 mL of YEM media), for 5–7 days (OD_{620}~0.8–1.0), as above. At this stage, 0.25 L of 50 μM genistein (in methanol) were added to each 250 mL of bacterial subculture (genistein concentration of 5 μM) and the culture was incubated for 48–96 hours, the flavone thereby inducing LCO production in the bacterial cells.

EXAMPLE 2

Induction of LCO production by *Rhizobium meliloti* or *Rhizobium leguminosarum*

The first culture of *Rhizobium meliloti* strain RCR2011 was grown at 28° C. in 100–125 mL of sterile yeast mannitol media (YEM) with pH 6.8, shaken at 150 rpm until the OD_{620} reaches 0.4–0.6 (2–3 days). Thereafter, a 2L bacterial subculture was started by inoculating first culture (5 mL of first culture per 250 mL of YEM media), for 2–3 days (OD_{620}~0.8–1.0), as above. At this stage, 0.25 mL of 50 μM luteolin (in methanol) was added to each 250 mL of bacterial subculture (luteolin concentration of 5 μM) and the culture was incubated for 48 hours, the flavone thereby inducing LCO production in the bacterial cells.

For LCO production by *Rhizobium leguminosarum*, the rhizobia was grown similarly as above. The flavone (naringenin) was added to the subculture of *R. leguminosarum* (10 μM) and the procedure carried out as above.

EXAMPLE 3

Extraction and Purification of LCOs

Two liters of bacterial subculture were phase-partitioned against 0.8 L of HPLC-grade 1-butanol by shaking overnight. The upper butanol layer was then transferred to a 1 L evaporation flask and evaporated at 80° C. to 2–3 mL of light brown, viscose material with a Yamato RE500 Rotary Evaporator, which was resuspended in 4 mL of 18% acetonitrile and kept in the dark at 4° C. in a sealed glass vial.

HPLC analysis was conducted with a Vydac C18 reversed-phase column with flow rate 1.0 mL/min and a Vydac guard column. As a baseline, acetonitrile (AcN/H_2O; w/w) was run through the system for at least 10 min. When the sample was loaded, an isocratic elution was started by 18% of AcN for 45 min. This step aims at removing all non-polar contaminant light fractions. Thereafter, a gradient elution for 90 min. with 18–82% AcN was performed. LCOs began to elute after 94–96 min. of HPLC run time.

For the purification of LCOs from *R. leguminosarum* (which nodulates numerous legumes), the HPLC peaks were identified and compared to those obtained with *B. japonicum* and *R. meliloti*. LCO peaks which were different from those of these two other rhizobia were identified collected. Thus, it is strongly suggested that the *R. leguminosarum* LCOs used herein are different from that of *B. japonicum* or *R. meliloti*.

EXAMPLE 4

Effect of LCO on Emergence of Some Plant Species

Plastic pots (7.5 cm dia) were filled with 15 g of autoclaved vermiculite. Seeds of corn (*Zea mays*—Poaceae), bean (*Phaseolus vulgaris*—Fabaceae), canola (*Brassica napus*—Brassicaceae), cucumber (*Cucumis sativus*—Cucurbitaceae), cantaloupe (*Cucumis melo*—Cucurbitaceae), cotton (*Gossypium* sp.—Malvaceae), lettuce (*Lactuca sativa*—Asteraceae), beet (*Beta vulgaris*—Chenopodiaceae), and soybean (*Glycine max*—Fabaceae), were placed at 2.5 cm deep at the rate of 5 or 10 seeds per pot. Pots were irrigated with either 25 mL of LCO solution at different concentrations (10^{-6}–10^{-14}M) or aqueous acetonitrile or water, as controls. Acetonitrile was included as one of the controls since LCO was purified in this solvent (see Example 3), after 4 days the pots were irrigated with 10 mL of water once every two days. Each treatment had 4 replications in a randomized block design. Pots were placed on a green house bench maintained at 25±2° C. with a day/night cycle 16/8 h and relative humidity of 70%, or in a growth chamber set at 15° C. with a 16:8 day/night cycle.

As defined above, seed germination has occurred when clear evidence of root growth developping from the embryo on the seed is observed.

As the time required for seedling emergence of the species used in the experiment varied considerably, observation on seedling emergence was recorded when the emergence was observed for at least 50% in most of the treatments. As defined above, seedling emergence has occurred when growth of the plant can be observed above the rooting medium surface. The percent emergence was calculated. The data were analyzed with Statistical Analysis System, version 6.12 (SAS institute Inc. Cary, N.C., USA).

LCO treatment reduced the time required from sowing to emergence of a number of economically important plant species tested. Among the species tested, *Z. mays, L. sativa, B. vulgaris, P. vulgaris*, and *G. max* showed significant increases in emergence when treated with LCO at 25° C. (Table 1), while, *C. sativus* and *B. napus* showed similar effects at 15° C. (Table 2).

TABLE 1

Effect of lipo chitooligosaccharide on seedling emergence (%) at 25° C.

Treatment	*Zea mays*	*Beta vulgaris*	*Glycine max*	*Gossypium* sp.	*Cucumis melo*	*Letuca sativa*	*Phaseolus vulgaris*
Control	40 c[Ψ]	33 a	40 e	55 b	80 a	5 d	44 abc
10^{-6} M	76 ab	NT	65 d	88 a	NT	45 a	67 abc
10^{-7} M	68 abc	66.6	80 bc	66 ab	100 b	35 ab	89 a
10^{-8} M	84 a	NT	90 ab	88 a	NT	10 dc	78 ab
10^{-9} M	88 a	66 b	100 a	88 a	100 b	20 bcd	67 abc
10^{-10} M	84 a	NT	70 cd	88 a	NT	25 abcd	44 abc
10^{-11} M	68 abc	86 b	50 e	NT	100 b	26 abcd	22 c
10^{-12} M	48 abc	NT	80 bc	NT	NT	30 abc	33 bc
10^{-13} M	40 c	80 b	70 cd	NT	100 b	5 d	33 bc
10^{-14} M	40 c	NT	70 cd	NT	NT	10 d	33 bc

[Ψ]means with in the same column, followed by the same letter are not significantly different ($p \leq 0.05$) by ANOVA protected LSD test.
NT - Not tested

TABLE 2

Effect of lipo chitooligosaccharide on seedling emergence at 15° C.

Treatment	*Cucumis sativus*	*Brassica napus*
Control	60 c[Ψ]	32.5 c
Lipo chitooligosaccharide		
10^{-6}M	65 abc	35 bc
10^{-7}M	85 ab	32 c
10^{-8}M	80 ab	35 bc
10^{-9}M	70 abc	52 ab
10^{-10}M	50 c	62 a
10^{-11}M	80 ab	47 bc
10^{-12}M	80 ab	45 bc
10^{-13}M	70 abc	37 bc
10^{-14}M	70 abc	30 c

[Ψ]means with in the same column, followed by the same letter are not significantly different ($p \leq 0.05$) by ANOVA protected LSD test.

In some plants, germination/emergence promoting effects of LCOs is seen at all temperatures suitable for growth, while in others, it is only observed under temperature-limiting conditions.

EXAMPLE 5

Effects of LCO on Early Growth of Corn

The percent seedling emergence was recorded at 4 days after sowing (DAS). Plant height was recorded from 4 DAS to 15 DAS. Plants were harvested at 15 DAS and leaf area, root length and the number of roots per plant were recorded. The plants were then dissected and placed in paper covers and dried at 90° C. for 24 h and the dry weights of roots, shoots and the spent seeds recorded. The data were analyzed with the Statistical Analysis System version 6.12 (SAS institute Inc. Cary, N.C., USA).

In LCO treatments, seedling emergence started 2–3 days after seeding while in the control it was 3–4 days. LCO treatments significantly increased leaf area, root length, number of roots, shoot dry weight and root dry weight, while the weight of spent seed recorded significant decreases as compared to the control (Table 3). The optimum effect was observed at an LCO concentration of 10^{-8}M. The decrease in spent seed weight is attributed to the rapid translocation of stored reserve from the seed endosperm to the embryo. Of interest, a dramatic increase of α-amylase activity was observed in the treated seeds.

TABLE 3

Effect of lipo chitooligosaccharide on early growth (after seedling emergence) of *Zea mays*

Treatment	Leaf area (cm^2)	Root length (mm)	No. of roots	Plant height at 15 DAS (mm)	Root dry wt./plant (mg)	Spent seed dry wt. (mg)	Shoot dry wt./plant (mg)
Water	5.81 f[Ψ]	103.1 e	5.1 e	79.8 d	53.2 de	163.6 a	28.8 e
Acetonitrile	7.63 ef	106.3 e	5.8 de	94.1 cd	65.4 e	147.4 ab	30.1 de
LCO 10^{-5}	11.7 cd	137.0 cd	6.8 cd	102.9 c	75.7 cd	123.5 bc	41.6 bcd
LCO 10^{-6}	18.2 a	150.8 bc	7.66 abc	130.4 ab	85.1 bc	90.7 cd	51.4 b
LCO 10^{-7}	15.7 ab	153.9 b	8.0 ab	130.1 ab	96.5 ab	99.5 cd	51.6 b
LCO 10^{-8}	19.3 a	187.0 a	8.4 a	142.6 a	103.4 a	76.3 d	66.2 a
LCO 10^{-9}	17.0 ab	144.4 cbd	7.75 abc	134.7 ab	83.3 bc	93.7 cd	46.8 bc
LCO 10^{-10}	13.9 bc	149.1 bc	7.95 ab	127.1 b	89.5 abc	89.8 cd	51.4 b
LCO 10^{-11}	8.51 def	132.1 d	6.69 cd	103.3	74.6 cd	115.9 bc	38.0 cde
LCO 10^{-12}	8.0 def	137.9 cd	7.25 d	98.3 c	84.6 bc	105.4 od	40.6 bcde

TABLE 3-continued

Effect of lipo chitooligosaccharide on early growth (after seedling emergence) of *Zea mays*

Treatment	Leaf area (cm^2)	Root length (mm)	No. of roots	Plant height at 15 DAS (mm)	Root dry wt./plant (mg)	Spent seed dry wt. (mg)	Shoot dry wt./plant (mg)
LCO 10^{-13}	10.4 cde	130.9 d	6.7 cd	91.8 cd	85.4 bc	109.6 cd	43.6 bc
LCO 10^{-14}	11.2 cde	136.8 cd	7.36 abc	99.9 c	90.2 abc	102.9 cd	46.6 bc

[w]means with in the same column, followed by the same letter are not significantly different ($p \leqq 0.05$) by ANOVA protected LSD test.

Taken together, Examples 4 and 5 and Tables 1–3 show that LCOs can stimulate seedling emergence in all tested plants. In addition, a significant growth stimulation of corn was observed. Furthermore, the spent seed weight results suggested that LCOs also had an effect on seed germination for all tested plants. The growth-promoting effect of LCOs on corn, a plant quite distantly related to legumes (i.e. corn is a monocot), strongly suggests that plants in general should show the same growth-responses to LCO treatment.

EXAMPLE 6

Dormancy Breaking Activity of LCO on Potato Mini Tubers

Signal solution is a bacterial fermentation tank product, comprising approximately 10^{-4} M LCO from *B. japonicum*. More specifically, signal solution is the supernatant from a culture of *B. japonicum* in which genestein (a flavone) had been introduced to promote LCO expression. Following the subculture of *B. japonicum*, the bacteria was removed. While the stimulatory effect of Signal solution in the soybean-*Bradyrhizobium* japonicum complex has been described (Zhang and Smith, 1995; Zhang et al. 1996), the effects of these plant substances in other plant species and their associated rhizospheres' organisms have not been investigated.

Gibberellic acid (GA) and kinetin affect both the germination rate and the percent germination of crop seeds.

Some studies have indicated that plant growth regulators (PGRs), such as gibberellic acids (GAs), stimulate seed germination at low temperatures. Durrant and Mash (1991) reported that adding gibberellins ($GA_{4/7}$) to sugar-beet seeds (*Beta vulgaris* L. Var. *altissima*) was beneficial to seed germination under cold, wet conditions.

Kepczynski and Bialecka reported that Methyl jasmonate (JA-Me) inhibited or retarded germination of *Amaranthus caudatus* seeds in darkness at 24° C. Ethephon, ACC (1-aminocyclopropane-1-carboxylic acid) and gibberellins (GA_3 or GA_{4+7}) partially or completely reversed this inhibition depending on the concentration of JA-Me applied.

Indeed, gibberellic acid, as well as bromoethane, are used commercially to break dormancy and to stimulate sprout formation.

Treatments were carried out on microtubers (200–400 mg) that had been cold-stored for 8 wk to determine their effect on breaking dormancy. Signal solution was used at full strength (100%) or diluted to 20% (as for soybean), 12%, or 6% of full strength. GA_3 (500 mg l^{-1}), water soaking, and control treatments were performed for comparison purposes. Microtuber soaking treatments lasted 24 h and then incubation occurred either in the light (40 μmol $m^{-2}s^{-1}$ cool-white fluorescent) or in the dark. Five microtubers were used in each treatment. Observations for sprouted microtubers were made at 1 and 2 wk.

One hundred % signal solution was as effective as GA_3 (500 mg l^{-1}) when evaluated after 1 wk with respect to the number of sprouted microtubers. Table 4 shows the effect of signal solution (SS) on dormancy breaking of potato microtubers as compared to the known dormancy breaker gibberellic acid (GA_3) (200–450 mg) that had been cold-stored at 5° C. for 8 weeks and evaluated after treatment and incubation with or without light for 1 and 2 weeks, for number of sprouts and for number with multiple sprouts (>1) at 2 weeks. One hundred % signal solution induced multiple sprouts and dark incubation favoured sprouting as compared with the light regime after 1 wk of incubation. The exact cause of dark incubation favouring 100% signal solution is not understood. One hundred % signal solution was more effective than diluted signal solution when numbers of sprouted microtubers were counted after 1 wk. After 2 wk of incubation all treatments were equally effective in causing sprouting but the signal solution and GA_3 solutions were most promotive of multiple sprouting which did not occur in the water soaking treatment and only in the control treatment incubated in the dark.

TABLE 4

Effects of signal solution (SS) on dormancy breaking of potato microtubers as compared to the known dormancy breaker gibberellic acid (GA_3)

Treatments	Number of sprouted microtubers 1 wk	Number of sprouted microtubers 2 wk	Number of multiple sprouted microtubers (2 wk)	Mean number of sprouts ± SE
GA_3 500 mgl^{-1} + light	1/5	5/5	2/5	2.5 ± 0.5
GA_3 500 mgl^{-1} – light	5/5	5/5	4/5	2.5 ± 0.3
100% SS + light	0/5	5/5	2/5	2.5 ± 0.5
100% SS – light	5/5	5/5	3/5	2.3 ± 0.3
20% SS + light	0/5	4/5	0	0
20% SS – light	2/5	5/5	2/5	2.0 ± 0
12% SS + light	0/5	4/5	1/4	2.0 ± 0
12% SS – light	2/5	5/5	2/5	2.0 ± 0
6% SS + light	0/5	4/5	2/4	2.0 ± 0
6% SS – light	1/5	5/5	2/5	2.0 ± 0
Water + light	0/5	4/5	0	0
Water – light	2/5	5/5	0	0
Control + light	0/5	5/5	0	0
Control – tight	1/5	5/5	1/5	2.0 ± 0

Tuber sprouting of potatoes is somewhat comparable to seed germination in the sense that the plant meristems are activated and the plant is beginning to grow, following quiescence. A signal molecule involved in bacteria-legume signalling was shown to be effective in breaking the dor-

mancy of a plant (potato) that is distantly related to the legumes. LCOs therefore seem to have a broad effect on breaking the dormancy or quiescence of plants.

EXAMPLE 7

Effects of Combinations of GA_3 and 100% Signal Solution in Breaking Dormancy of Potato Tubers

The data presented in Example 6 suggested that the 100% signal solution (and thus the LCO purified from *B. japonicum*) was effective in breaking microtuber dormancy. However, the microtubers used in the trial had been cold-stored for 8 wk. In this trial, the effects of signal solution were evaluated in combination with GA_3 on minitubers with only 3 wk of cold storage. It was also investigated whether the effect of 100% signal solution might be synergistic if used with GA_3 (500 mg l^{-1}).

Minitubers (20–30 g) with 3 wk cold storage treatment were soaked for 24 h in 500 mg l^{-1} GA_3, 100% signal solution, or a mixture of the two. Another treatment involved successive soaking for 12 h each, in first GA_3, and then signal solution. A control treatment without soaking was also performed. Eight minitubers were used per treatment which were applied at room temperature (20° C.). Microtubers were observed after 2 wk and the number of sprouted minitubers and the number with multiple sprouts were counted.

All treatments were able to break minituber dormancy except the control (Table 5). The 500 mg 11 GA_3 treatment alone or together with 100% signal solution, for 24 h, caused 100% sprouting, and significantly more multiple sprout formation than the other treatments. The 100% signal solution alone or in combination with 500 mg^{-1} GA_3 were as effective as 500 mgl^{-1} GA_3 alone for breaking dormancy within 2 wk. However, less multiple sprouting occurred with 100% signal solution alone, or following 12 h GA_3 treatment, compared with the GA_3 treatment alone or the combined GA_3 and Signal treatments. When working under the conditions tested, there were no clear synergistic effects of 100% signal solution in combinations with 500 mg l^{-1} GA_3 on the number of sprouted tubers or multiple sprouts.

TABLE 5

Individual and combined effects of GA_3 and 100% signal solution (SS) on dormancy breaking of minitubers that had been cold-stored for 3 weeks

Treatments	Number of sprouted minituber	Number of minituber with multiple sprouts	Mean number of sprouts ± SE
GA_3 500 mgl^{-1} 24 h	8/8	6/8	3.37 ± 0.62
100% SS 24 h	7/8	2/7	1.33 ± 0.42
GA_3 500 mgl^{-1} + 100% SS 12 h + 12 h	8/8	6/8	2.37 ± 0.41
GA_3 500 mgl^{-1} + 100% SS Combination 24 h	8/8	6/8	3.75 ± 0.67
Control	0/8	0/8	0/8

These results suggest that bacteria-legume signal molecules are effective in breaking the dormancy of potatoes. Taken together with the results presented above (i.e. Example 4 and Table 1) showing the effects of a pure LCO in breaking the quiescence of seeds and promoting growth of a variety of distantly related plants, strongly supports the contention that LCOs are effective at breaking the dormancy of potatoes and promoting the activity of plant meristems in general.

EXAMPLE 8

Effectiveness of GA_3 and Signal Solution, as Compared to Bromoethane and Mechanical Injuries, in Breaking Dormancy

Bromoethane (BE) was reported to break potato tuber dormancy when applied as a fumigant and it was found that BE at a concentration of 0.2 ml l^{-1} was the most effective (Coleman, 1983). Conventionally, large potato tubers are cut into small pieces, each containing an eye, to be used as seed pieces. To obtain quick and uniform sprout emergence, potato tubers should be cut at least 2 wk before planting (Slomnicki and Rylski, 1964). Mechanical injury is also shown to contribute to sprout induction. The objective of this experiment was to compare the effects of known dormancy-breaking treatments on microtubers and minitubers 1) BE; 2) GA_3; and 3) mechanical injury; with the newly identified dormancy breaker: LCOs.

Microtubers (200–500 mg) cold-stored for 8 wk and minitubers (20–35 g) cold-stored for 0, 2, or 8 wk were used for these experiments. BE (0.2 ml l^{-1}) and mechanical injury (cutting in half, microwaving at full power for 10 sec) were compared with GA_3 (500 mgl^{-1}), 100% signal solution, water soaking, and control treatments. Six microtubers or minitubers were used per treatment. Observations were made at 1, 2, 3, and 4 wk intervals and the number of sprouted tubers were counted. The evaluation period was extended because tubers with little or no cold storage treatment took longer to sprout.

GA_3 was the only agent which was able to break dormancy of minitubers that had not been cold-stored; 0/6 at 2 wk but 4/6 by 4 wk (minituber, 0 wk storage; Table 6). Minitubers with 2 wk cold storage that were treated with GA_3 also broke-dormancy; 0/6 at 2 wk but 5/6 by 4 wk with 216 showing multiple shoots. Signal solution treatment of minitubers cold-stored for 2 wk caused 1/6 (with multiple shoots) minitubers to break dormancy after 4 wk. For minitubers cold-stored for 8 wk, all treatments (including water soaking and control), except the microwaving, showed some sprouting the first week and multiple sprouting was evident in the GA_3 (6/6), 100% signal solution (4/6), and BE (1/6) treatments by 2 wk. Cutting caused sprouting in 9/12 cut halves by 2 wk but only a few multiple shoots (2/12) were evident by 3 wk.

TABLE 6

Sprouting and (multiple sprouting) performance on minitubers and microtubers cold-stored for 0, 2, 4, or 8 weeks after exposure to Bromoethane (0.2 ml l^{-1}), mechanical injury (cutting in half or microwaving), GA_3 (500 mg l^{-1}), 100% signal solution, water soaking, and control treatments.

	Minituber (CS8W)				Minituber (CS2W)				Minituber (NCS)				Microtuber (CS8W)			
Treatments	1 wk	2 wk	3 wk	4 wk	1 wk	2 wk	3 wk	4 wk	1 wk	2 wk	3 wk	4 wk	1 wk	2 wk	3 wk	4 wk
GA_3 500 mg l^{-1}	5/6	6/6 (6)	6/6 (6)	6/6 (6)	0	0	3/6	5/6 (2)	0	0	3/6	4/6	4/6	5/6 (3)	6/6 (3)	6/6 (6)
100% Signal solution	4/6	6/6 (4)	6/6 (4)	6/6 (4)	0	0	0	1/6 (1)	0	0	0	0	1/6	2/6	4/6/	6/6 (1)
Bromoethane 0.2 ml l^{-1}	3/6	4/6 (1)	6/6 (4)	6/6 (4)	0	0	0	2/6	0	0	0	0	2/6	4/6	4/6	6/6
Cutting into halves	7/12	9/12	12/12 (2)	12/12 (2)	0	0	0	1/12	0	0	0	0	5/12	8/12	9/12	10/12
Microwaving	0	0	1/6	2/6	0	0	0	0	0	0	0	0	0	0	0	1/6
Water	4/6	5/6	6/6 (1)	6/6 (1)	0	0	0	0	0	0	0	0	2/6	4/6	6/6	6/6
Control	2/6	4/6	6/6	6/6	0	0	0	0	0	0	0	0	3/6	4/6	6/6	6/6

'CS' stands for cold storage;
'W' for week;
N for no
'( )' denotes number of tubers with multiple sprouts

With microtubers cold-stored for 8 wk, dormancy-breaking occurred within the first week in all treatments except microwaving. After 2 wk, sprouting had progressed in all treatments; water (4/6) was similar to GA_3 solution (5/6), BE (4/6), signal solution (416), and cutting (8/12). Only GA_3 solution (3/6 at 2 wk, 6/6 at 4 wk) and signal solution (1/6 at 4 wk) caused multiple sprout formation.

Signal solution (100%) was effective in causing sprouting in minitubers with 2 or 8 wk cold storage and microtubers with 8 wk cold storage but it was ineffective on minitubers that had not been cold-stored. GA_3 and 100% signal solution induced multiple sprouts from different eye-points unlike the control BE, or cutting treatments, that induced single sprouts only from the rose end. BE worked well in inducing single sprouts in minitubers and microtubers with 8 wk cold-storage but was not as effective as GA_3 for minitubers that had not been cold-stored.

Cutting minitubers or microtubers in half after 8 wk cold storage induced single sprouts on each cut half. This occurred quite efficiently in minitubers (9/12 in 2 wk, 12/12 in 4 wk) and somewhat less efficiently with microtubers (8/12 in 2 wk, 10/12 in 4 wk). Cutting was not effective in breaking dormancy in minituber without cold storage and worked poorly in minitubers that had been cold stored for only 2 wk (0/12 after 2 wk, 1/12 after 4 wk). Sprouting from two halves was good, in the sense that by cutting minitubers or microtubers in half, two propagules, each with one sprout were derived although a very insignificant number of minituber halves (2/12) showed multiple sprouting. However, cutting was risky in that this sometimes provided opportunities for fungal or bacterial infection. Microwaving induced limited sprouting but only in minitubers or microtubers that had been cold-stored for 8 wk, and not in the minitubers with 0 or 2 wk cold storage. Microwaving caused some tuber damage that may account for the reduced sprouting observed.

In short, signal solution was effective in promoting both sprouting and multiple sprouting of mini- and micro-tubers and, of the tested treatments, only GA_3 was better.

EXAMPLE 9

Effectiveness of Anti-ABA Compared with Other Dormancy Breaking Treatments

Anti-Abscisic acid (anti-ABA), the acetylenic analog of ABA, has never been used to induce sprouting in dormant potato tubers since it was first shown to be an ABA antagonist (Wilen et al., 1993). However, anti-ABA has been used to terminate dormancy in canola seeds (PBI Bulletin, 1995). The objective of this experiment was to test anti-ABA for breaking dormancy in potato minitubers and compare its efficacy with other dormancy-breaking treatments.

Microtubers (200–600 mg) were cold-stored for 3 wk prior to the experiment. Seven microtubers were used per treatment. Treatments included 24 h soaks in anti-ABA or GA_3 (500 and 250 mg l^{-1}, respectively) applied alone or in combination, GA_3 (500 mg l^{-1} in combination with 100% signal solution, and water. Bromoethane (0.2 ml l^{-1}) and control treatments were also performed. Observations were made after 2 wk in the dark at room temperature (20° C.). Data included number of sprouted microtubers and number of multiple sprouts. Means of sprout number were calculated only from microtubers that had sprouted.

Anti-ABA alone and in successive treatments or in combination with GA_3 was effective in breaking microtuber dormancy (Table 7). Among the different treatments using anti-ABA and GA_3 the greatest mean number of sprouts (1.8±0.48) occurred when microtubers were soaked in a mixed solution of 500 mg l^{-1} GA_3 and 500 mg l^{-1} anti-ABA for 24 h but it was not significantly different from the 500 mg l^{-1} GA_3 treatment (1.71±0.28). The combined signal solution and GA_3 was not more effective than GA_3 alone and was less effective than any GA_3 and anti-ABA treatment in breaking dormancy.

TABLE 7

Sprouting performance on microtubers cold-stored for 3 weeks and evaluated at 2 weeks after exposure to dormancy-breaking agents.

Treatments	No. sprouted microtubers	Mean No. sprouts ± SE
GA_3 500 mgl^{-1} 24 h	7/7	1.71 ± 0.28
GA_3 500 mgl^{-1} + 100% SS 24 h	3/7	1.33 ± 0.29
Anti-ABA 500 mgl^{-1} 24 h	5/7	1.4 ± 0.24
Anti-ABA 250 mgl^{-1} 24 h	5/7	1.2 ± 0.20
GA_3 500 mgl^{-1} 12 h + Anti-ABA 500 mgl^{-1} 12 h	6/7	1.33 ± 0.21
GA_3 250 mgl^{-1} 12 h + Anti-ABA 250 mgl^{-1} 12 h	7/7	1.57 ± 0.29
GA_3 500 mgl^{-1} + Anti-ABA 500 mgl^{-1} Combination 24 h	5/7	1.8 ± 0.48
GA_3 250 mgl^{-1} + Anti-ABA 250 mgl^{-1} Combination 24 h	5/7	1.6 ± 0.24
Bromoethane	1/7	1.0 ± 0
Water	1/7	1.0 ± 0
Control (no treatment)	0/7	0

The overall results with anti-ABA underline its importance as a potential dormancy-releasing agent, as much so as GA_3 Anti-ABA and GA_3 both induced multiple sprouts but sprouts were longer after GA_3 than anti-ABA treatment. Both agents caused sprouts to emerge at various eyes over the tuber surface. However, the GA_3-induced multiple sprouts were profusely branched; a group of sprouts protruded from each eye, while the anti-ABA-induced sprouts were singles. The mechanism of dormancy breaking by anti-ABA and GA_3 therefore was similar, but GA_3 appeared stronger. These agents should be tested on an equimolar basis in the future.

EXAMPLE 10

Harvests from Minitubers Sprouted Using a Range of Dormancy-Breaking Treatments

There is only limited information on the relative yield performance of potato tubers that were treated with dormancy-breaking agents (Choudhury and Ghose, 1960; Slomnicki and Rylski, 1964). Yields from potato tubers that were treated with GA_3 at 25–100 mg l^{-1} (Choudhuri and Ghose, 1960) or 5–40 mg l^{-1} (Slomnicki and Rylski, 1964) were reduced compared with untreated controls. The objective of this experiment was to evaluate the effect of dormancy-breaking agents on subsequent yield in greenhouse pot trials.

Minitubers (20–35 g) that were cold-stored for 8 wk were given dormancy-releasing treatments including 24 h soaking in GA_3 (500 mg l^{-1}), 100% signal solution, or water. Other treatments included BE (0.2 m l^{-1}), cutting in half, and the control. All minitubers were observed at 3 wk following treatment and the number of sprouts were noted at the time of planting. Five minitubers per treatment were individually planted into 11×12 cm plastic pots in the greenhouse. The potting mixture was 2:1 peat:perlite without fertilizer added. The pots were arranged in a complete randomized design and watered equally every alternate day. Harvest occurred after 60 d and tuber yields (number and fresh weight) were recorded.

GA_3 caused significantly more sprouts per minituber (4.2±0.37) than the other treatments, with 100% signal solution (2.0±0.31) and BE (1.8±0.2) giving intermediate values, and water-soaking and cutting similar to the control (Table 8). The average number of tubers per plant was greatest in the GA_3 treatment (3.6); almost double that of other treatments that were not different from the control. Suprisingly, however, the mean fresh weight of tubers (per replicate i.e. pot basis) harvested from minitubers exposed to the 100% signal solution treatment was the greatest (34.97 g); greater than the control fresh weight and three times more than the GA_3 treatment. The size and shape of tubers harvested from the GA_3 treatment were small and more elongated than that of the control and other treatments. Yields from BE treated minitubers were significantly lower compared with controls. The cut halves each yielded almost the same as uncut controls and had similar fresh weight to control (28.41 vs 27.16). Two cut halves of each minituber together would effectively double control yield and bring the mean number of tubers into the GA_3 treatment range. However, cutting into halves posed a problem of infection and decomposition at the cut surfaces.

Thus, although signal solution is not as efficacious as GA_3 in breaking dormancy (as evaluated by the number of sprouts), it however is significantly more efficient than GA_3 in increasing the tuber yield. LCOs therefore appear as the best agents to promote dormancy breaking and yield increases in potato.

TABLE 8

Harvests after 60 d from minitubers that were forced to break dormancy by different methods.

Treatments	Mean number sprouts at planting ± SE	Mean number of tubers produced per replicate	Mean fresh weight (g)
GA_3	4.2 ± 0.37	3.6*	11.13 d
Culling into halves (½ minituber)	1.2 ± 0.20	2.0	28.41 b
Bromoethane	1.8 ± 0.20	2.0	14.01 d
100% Signal soln.	2.0 ± 0.31	1.8	34.97 a
Water	1.2 ± 0.20	1.0	17.41 c
Control	1.0 ± 0	2.0	27.16 b

Numbers represented by the same letter are not significantly different at the 0.05 level.

It shall be recognized therefore that agricultural compositions comprising at least one LCO and gibberellic acid (GA_3 and others known in the art) could be advantageously used in accordance with the methods of the present invention to break dormancy and/or quiescence of crop plants.

EXAMPLE 11

Other LCOs

Following the methods described above, the LCO most abundantly produced by *R. meliloti* (Nod Rm-V($C_{16:2}$, S)) was isolated and tested on alfalfa (*Medicago sativa*) seeds. Briefly, 10 seeds were placed in a disk of filter paper on a petri plate. The filter paper was wetted with 5 ml of the appropriate LCO solution. Data were taken at 12 hour intervals upon the radicle (an embryonic root). The number of seed with an emerged radicle were counted. Each treatment was repeated four times. The data presented in table 9 indicate a clear acceleration of growth. In this case no

standard for HPLC calibration was available, so a relative dilution series was used. In addition, a cluster of peaks specifically induced by the specific flavone of *Rhizobium leguminosarum* (*bv phaseoli* [strain 127K105]) were collected and tested on corn (*Zea mays*), red clover (*Trifolium repens*, Fabaceae) and pea (*Pisum sativum*, Fabaceae) (Table 10). In each case, a stimulation of seed germination was observed. Of note, *Rhizobium leguminosarum* produces a large number of LCOs. A subset of these LCOs was selected from a range of the HPLC profile where the LCOs from *B. japonicum* and *R. meliloti* did not occur. Taken together, these results clearly demonstrate that the promoting effects of LCOs on plant growth disclosed herein are observable with LCOs from different bacterial strains involved in bacteria-legume signalling. Consequently, the presented data strongly suggests that LCOs in general should demonstrate the same effects on seed germination, seedling emergence, growth, dormancy breakage and the like.

TABLE 9

Effect of LCO isolated from *Rhizobium meliloti* (RCR 2011) on germination of alfalfa after 24 h of treatment

Treatment	Percent Germination
Control	16.7 b
10^{-1} dilution	26.6 ab
10^{-2} dilution	26.6 ab
10^{-3} dilution	36.6 a
LSD ($p < 0.05$)	19.2

In column numbers followed by same letter do not differ significantly by an ANOVA protected LSD test at $p < 0.05$

TABLE 10

Effect of LCOs of *Rhizobium leguminosarum* bv *phaseoli* (strain 127K 105) on seed germination (%) of corn (after 48 h), red clover (after 12 h) and pea (after 48 h) at 25° C.

Treatment	Corn	Red Clover	Pea
Control	20 a	43.3 bc	26.6 b
10^{-1} dilution	26.6 a	26.6 c	26.6 b
10^{-2} dilution	60.0 b	63.3 ab	20.0 b
10^{-3} dilution	20.0 a	66.6 a	73.3 a
LSD ($p < 0.05$)	19.9	23.3	29.7

In column numbers followed by same letter do not differ significantly by an ANOVA protected LSD test at $p < 0.05$

EXAMPLE 12

Germination Versus Emergence

Seeds of corn (cv Pioneer 3921) were surface sterilized in 2% sodium hypochlorite solution for 2 minutes and placed in 9 cm diameter Petri plates containing a sheet of filter paper soaked in 10 ml of the required test solution (LCO 10^{-5}–10^{-13}). Water served as the control. Observations on germination, length of root primodia and shoot were taken after 72 h of incubation at 25° C. The data was analyzed for significance by an ANOVA protected LSD test using SAS system Version 6.1 (SAS Inc., Cary, N.C., USA).

TABLE 11

Effect of lipo chitooligosaccharide [Bj Nod-V ($C_{18:1}$ MeFuc)] on germination of corn (*Zea mays* L.) after 72 h of treatment

Treatment	Percent germination	Length of root primodia (mm)	Length of shoot primodia (mm)
Control	46.6 a	32.3 a	4.6 a
LCO 10^{-5} M	80 bc	53.0 ab	12.3 ab
LCO 10^{-7} M	73.3 b	57.6 bc	15.0 ab
LCO 10^{-9} M	73.3 b	48.0 ab	9.6 a
LCO 10^{-11} M	100 c	78.6 c	21.0 b
LCO 10^{-13} M	80 bc	43.0 bc	8.3 a
LSD ($p < 0.05$)	22.6	24.3	11.0

In column numbers followed by same letter do not differ significantly by an ANOVA protected LSD test at $p < 0.05$.

Table 11 shows that incubation of corn seeds with LCO solution significantly improved the germination of corn and increased the length of both shoot and roots.

EXAMPLE 13

Seedling Emergence-Promoting Effects of LCOs Under Field Conditions

Seeds of corn, cotton, beet, and soybean which showed promising results under laboratory conditions were tested for seedling emergence under field condition. Seeds were surface sterilized with 2% sodium hypochlorite and soaked in different concentrations (10^{-5}, 10^{-7}, 10^{-9}M) of LCO solution for 12 h. Water served as the control. The study was conducted at the experimental field of the Macdonald campus of McGill University, Ste-Anne-de-Belleveue. Quebec, Canada. The field was ploughed to a fine tilth, seeds were hand planted in 1 m rows at 2.5 to 3 cm deep with three replications per treatment. The percent seedling emergence was observed at six days after planting during which time at least 50% of the seeds emerged in the treatments. The data was analyzed for significance by an ANOVA protected LSD test using SAS system Version 6.1 (SAS Inc., Cary, N.C., USA).

TABLE 12

Effect of lipo chitooligosaccharide [Bj Nod-V ($C_{18:1}$ MeFuc)] on seedling emergence under field condition

Treatment	Corn	Cotton	Beet	Soybean
Control	41.6 a	6.6 a	26.6 a	16.6 a
LCO 10^{-5} M	80.0 b	16.6 a	28.3 a	26.6 ab
ICO 10^{-7} M	60.0 b	60.0 b	46.6 c	33.3 b
LCO 10^{-9} M	53.3 b	23.3 a	38.3 ab	63.3 c
LSD ($P < 0.05$)	35.7	28.0	16.3	16.6

In column numbers followed by same letter do not differ significantly by an ANOVA protected LSD test at $p < 0.05$

Table 12 shows that LCO [Bj Nod-V ($C_{18:1}$ MeFuc)] treatment enhanced the seedling emergence under field conditions of all the crop species studied. The best effect was observed in cotton where LCO at 10^{-7}M improved ±5 the emergence by more than 9 times as compared to the control. The effective concentration of LCO varied with the species.

Table 12 also validates the laboratory results presented herein by demonstrating that the stimulatory effects of LCOs are operating on four different crops under field conditions.

Thus the present invention provides agricultural compositions and methods by which LCO could be used to enhance

the germination, seedling emergence, root growth and improve early growth of crops under laboratory or field conditions.

CONCLUSION

The present invention therefore provides evidence that, among other things: (1) lipo chitooligosaccharide (LCO) treatment enhances the seedling emergence of higher plant seeds (egs. *Z. mays, L. sativa, B. vulgaris, P. vulgaris, G. max, C. sativus, B. napus* and *M. sativa*); (2) lipo chitooligosaccharide breaks the dormancy of potato (*Solanum tuberosum*) minitubers and increases their yield; (3) lipo chitooligosaccharide improves emergence and early growth, including root growth, of *Z. mays* giving a competitive advantage over non treated ones; (4) lipo chitooligosaccharide enhances the translocation of stored seed reserve; and (5) lipo chitooligosaccharide enhances seed germination.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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What is claimed is:

1. A method for enhancing seed germination or seedling emergence of a plant crop comprising the steps of:

providing a composition that comprises an effective amount of at least one lipo chitooligosaccharide (LCO) and an agriculturally suitable carrier; and

applying the composition in the immediate vicinity of a seed or seedling in an effective amount for enhancing seed germination or seedling emergence in comparison to an untreated seed or seedling.

2. A method for enhancing in a non-legume, seed germination, seedling emergence or growth of a plant crop comprising the steps of:

providing a composition that comprises an effective amount of at least one lipo chitooligosaccharide (LCO) and an agriculturally suitable carrier; and

applying the composition in the immediate vicinity of a seed, root or plant in an effective amount for enhancing seed germination, seedling emergence or growth of said plant in comparison to an untreated plant.

3. The method according to claim **2**, wherein said plant crop is selected from the group consisting of Poaceae, Cucurbitaceae, Malvaceae, Asteraceae, Chenopodiaceae, Solanaceae and Brassicaceae.

4. The method according to claim **3**, wherein said plant crop is selected from the group consisting of corn, cotton, cucumber, cantaloupe, lettuce, beet, canola and potato.

5. The method according to claim **1**, wherein said LCO is obtainable from a rhizobia selected from the group consisting of *Bradyrhizobium japonicum, Rhizobium meliloti* and *Rhizobium leguminosarum.*

6. The method according to claim **5**, wherein said LCO is present in said composition at a concentration of between about 10^{-5}M to about 10^{-14}M.

7. The method according to claim **5**, wherein said LCO is present in said composition at a concentration of between about 10^{-6}M to about 10^{-12}M.

8. The method according to claim **5**, wherein said LCO is present in said composition at a concentration of between about 10^{-7}M to about 10^{-10} M.

9. The method according to claim **1**, wherein said composition is effective in enhancing seed germination or seedling emergence under field conditions.

10. The method according to claim **1**, wherein said plant crop is a member of the Fabaceae family.

11. The method according to claim **10**, wherein said plant crop is selected from the group consisting of soybean, bean, alfalfa and clover.

12. The method according to claim **10**, wherein said LCO is obtainable from a rhizobia selected from the group consisting of *Bradyrhizobium japonicum, Rhizobium meliloti* and *Rhizobium leguminosarum.*

13. The method according to claim **12**, wherein said LCO is present in said composition at a concentration of between about 10^{-5} M to about 10^{-14} M.

14. The method according to claim **12**, wherein said LCO is present in said composition at a concentration of between about 10^{-6} M to about 10^{-12} M.

15. The method according to claim **12**, wherein said LCO is present in said composition at a concentration of between about 10^{-7} M to about 10^{-10} M.

16. The method according to claim **10**, wherein said composition is effective in enhancing seed germination or seedling emergence under field conditions.

17. A method for breaking the dormancy or quiescence of a plant comprising the steps of:

providing an agricultural composition comprising at least one lipo chitooligosaccharide (LCO) and an agriculturally suitable carrier; and

applying the composition in the immediate vicinity of a seed, tuber or root in an effective amount to enable a breaking of the dormancy or quiescence of the seed, tuber, or root, in comparison to an untreated seed, tuber, or root.

18. The method according to claim **17**, wherein said plant is a member of the family of Solonaceae.

19. The method according to claim **18**, wherein said plant is a potato.

20. The method according to claim **19**, wherein said growth-promoting activity of said composition enables an increase in yield.

21. The method according to claim **19**, wherein said composition further comprises gibberellic acid.

22. A method for enhancing seed germination or seedling emergence of a plant crop comprising the steps of:

providing a rhizobial strain that expresses a lipo chitooligosaccharide (LCO); and

incubating the rhizobial strain in the immediate vicinity of one of a seed or seedling of said plant such that said LCO enhances seed germination or seedling emergence in comparison to a non-inoculated seed or seedling.

23. A method for enhancing in a non-legume, seed germination, seedling emergence or growth of a plant crop comprising the steps of:

providing a rhizobial strain that expresses a lipo chitooligosaccharide (LCO); and

incubating the rhizobial strain in the immediate vicinity of one of a seed or root of said plant such that said LCO enhances seed germination, seedling emergence or growth of said plant crop, wherein said incubation enhances seed germination, seedling emergence or growth in comparison to a non-inoculated seed or root of said plant.

24. The method of claim **23**, wherein said plant crop is selected from the group consisting of Poaceae, Cucurbitaceae, Malvaceae, Asteraceae, Chenopodiaceae, Solanaceae and Brassicaceae.

25. The method of claim **24**, wherein said plant crop is selected from the group consisting of corn, cotton, cucumber, cantaloupe, lettuce, beet, canola and potato.

26. The method of claim **22**, wherein said rhizobia is selected from *Bradyrhizobium japonicum, Rhizobium meliloti* and *Rhizobium leguminosarum.*

27. The method of claim **22**, wherein said LCO enhances seed germination or seedling emergence under field conditions.

28. The method of claim **22**, wherein said plant crop is a legume in the Fabaceae family and wherein said LCO enhances seed germination or seedling emergence under field conditions.

29. The method of claim **17**, wherein said composition comprises a bacterial strain which expresses said LCO.

30. The method of claim **29**, wherein said bacterial strain is a rhizobial strain.

31. The method of claim **1**, wherein said composition comprises a bacterial strain that expresses said LCO.

32. The method of claim **31**, wherein said bacterial strain is a rhizobial strain.

33. A method for enhancing seed germination or seedling emergence of a plant crop comprising the steps of:

providing a bacterial strain that expresses a lipo chitooligosaccharide (LCO); and

incubating said bacterial strain in the immediate vicinity of one of a seed or seedling of said plant such that said LCO enhances seed germination or seedling emergence of said plant crop, wherein said incubation enhances seed germination or seedling emergence in comparison to a non-inoculated seed or seedling of said plant.

34. A method for enhancing seed germination or seedling emergence of a plant crop comprising the step of:

providing a bacterial strain that expresses a lipo chitooligosaccharide (LCO) in the immediate vicinity of one of a seed or seedling of said plant such that said bacterial strain, upon expression of said LCO, enhances seed germination or seedling emergence of said plant crop, in comparison to a non-treated seed or seedling of said plant.

35. The method of claim **34** wherein said bacterial strain is a rhizobial strain.

* * * * *

EXHIBIT D

US005175149A

United States Patent [19]

Stacey et al.

[11] Patent Number: 5,175,149

[45] Date of Patent: Dec. 29, 1992

[54] PENTASACCHARIDE PHYTOHORMONES AND METHODS FOR THEIR USE

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[21] Appl. No.: 771,356

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[52] U.S. Cl. 514/23; 514/25; 514/54; 514/55; 514/62; 536/53; 536/55.1; 536/55.2; 71/77

[58] Field of Search 536/53, 55.1, 55.2; 514/25, 54, 62, 55, 23; 71/77

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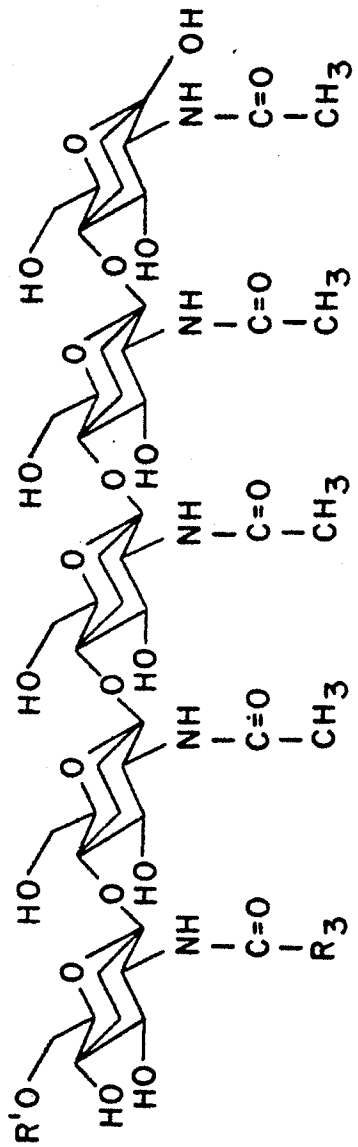
[57] ABSTRACT

A phytohormone for inducing root hair curling and root nodulation in the roots of leguminous plants particularly in the absence of nitrogen-fixing bacteria. The phytohormone comprises a pentassaccharide having a fatty acid condensed on the non-reducing end. a method for treating the roots of leguminous plants for inducing root hair curling and root nodulation is also disclosed.

6 Claims, 1 Drawing Sheet



Compound	R	R'	m+n
BJ Nod-V (C18:1)	$(CH_2)_n-CH=CH-(CH_2)_m-CH_3$		15
BJ Nod-V (Ac,C18:1)	$(CH_2)_n-CH=CH-(CH_2)_m-CH_3$	$CH_3CH_2C(=O)$	15
BJ Nod-V (C16:1)	$(CH_2)_{15}-CH_3$	H	—
BJ Nod-V (Ac,C16:0)	$(CH_2)_{15}-CH_3$	$CH_3CH_2C(=O)$	—
BJ Nod-V (C16:1)	$(CH_2)_n-CH=CH-CH_2-(CH_2)_m-CH_3$	H	13

Fig. 1

PENTASACCHARIDE PHYTOHORMONES AND METHODS FOR THEIR USE

The present invention relates to compounds and compositions for inducing changes in plants. The present invention also relates to methods for the application of those compounds and compositions to plants.

Phytohormones are compounds which induce changes in plants. Such compounds are useful in the communication between plants and other organisms. For example, many species of plants produce a phytohormone which is released from their roots into the soil. The hormone prevents the roots of plants of the same species from growing in that area. Therefore, plants of the same species in a limited area will have root systems that do not overlap.

Phytohormones are also released by organisms other than plants. Nitrogen-fixing bacteria, such as Rhizobium. Bradyrhizobium, and Azorhizobium species, release hormones in the vicinity of the roots of leguminous plants, such as soybean or alfalfa. Such hormones induce nodulation and root hair curling in the plants which make the plant roots suitable for symbiotic inhabitation by the bacteria. The bacteria and the plants then exist in a symbiotic relationship with the bacteria fixing nitrogen into the soil and the plants providing food to the bacteria.

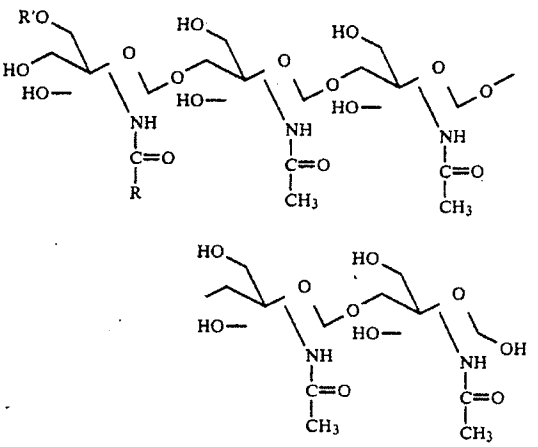
Generally, the nitrogen-fixing bacteria produce the root hair curling and root nodulation phytohormone in response to the production of flavonoids by the plants. Flavonoids comprise a large group of secondary products found in all higher plants. It is believed that flavonoids induce the expression of genes in the bacteria necessary to produce the phytohormone. The proteins believed to be involved in phytohormone production are encoded by the nod and nol genes. The phytohormone is then secreted and the plants respond with the production of nodules and curled root hairs.

In order to promote the effective inoculation of leguminous plants with specific strains of rhizobial bacteria, those strains are presently coated on the seeds or introduced into the soil of the plants. Soybean farmers sow seeds which are coated with those rhizobial strains which are known to be effective nitrogen-fixers. The soil may also be pretreated with the bacteria prior to sowing. In either case, the soybean seedlings rely solely on the ability of the particular strain of bacterium to induce root hair curling and root nodulation.

Although this process is more effective than natural processes for the inoculation of the plant, the rhizobial bacteria will often be insufficient to induce the desired level of nodulation. The desired particular strains of bacteria may be partially or totally supplanted by the natural rhizobial species in the soil or the desired strains may not be particularly efficient at inducing the production of nodules for the bacteria to inhabit. The mere coating of the leguminous seeds or sowing of the soil with the desired bacterial strains does not necessarily lead to the desired inoculation of the plant. Therefore, it is desirable to have a means for inducing nodulation on the roots of leguminous plants that is independent of the presence of rhizobial bacteria.

It has not been possible previously to induce root hair curling and root nodulation in leguminous plants in the absence of nitrogen-fixing bacteria. The present inventors have discovered and purified a phytohormone which is capable of inducing root hair curling and root

nodulation in the roots of leguminous plants. The phytohormone is a pentasaccharide of N-acetylglucosamine having a fatty acid condensed on the non-reducing end. The phytohormone of the present invention has the structure:



the pentasaccharide, R is a fatty acid condensate and R' is selected from the group consisting of H and an acetyl, $CH_3CH_2C{=}O$. The fatty acid is selected from the group consisting of $(CH_2)_{15}{-}CH_3$ and $(CH_2)_n{-}CH{=}CH{-}(CH_2)_m{-}CH_3$, where n +m is 13 or 15. These fatty acids are alternatively designated using the terms C16:0, C16:1 and C18:1.

In accordance with the method of the present invention root hair curling and root nodulation of the roots of leguminous plants are induced. The method comprises treating the roots of the plant with a pentasaccharide phytohormone having a fatty acid condensed on the non-reducing end. The pentasaccharide has the structure shown herein above and is applied to the plant root in a concentration of from about 10^{-12} M to about 10^{-3} M. At concentrations greater than about 10^{-3} M the hormone actually inhibits root hair curling and root nodulation. Thus, care must be taken when treating the roots of the selected leguminous plant with the phytohormone.

A drawing of the phytohormone is shown in FIG. 1. The depicted phytohormone is a pentasaccharide of N-acetylglucosamine. The non-reducing end of the pentasaccharide is substituted with a C18:1, C16:1, or C16:0 fatty acid. Further, the non-reducing sugar may be acetylated.

The phytohormone is named as follows: "BJ" indicates that the compound is isolated from *Bradyrhizobium japonicum;* "Nod" indicates that the compound is involved in nodulation; "-V" indicates that there are five N-acetylglucosamines in the compound structure. The terms in the parentheses indicate whether the compound is acetylated ("Ac") or not at the R' position and the structure of the fatty acid at the R position. The term C18:1 indicates an 18 carbon chain with one double bond; C16:0 indicates a 16 carbon chain with no double bond; and C16:1 indicates a 16 carbon chain with one double bond. Thus, the phytohormone BJ Nod-V (Ac, C18:1) is a nodulating pentasaccharide derived from *B. japonicum* which has been acetylated and contains an 18 carbon fatty acid residue with one double bond.

The phytohormone preferably is purified by recovery from *Bradyrhizobium japonicum* which has been induced to produce the phytohormone by the presence of flavonoids. The crude extracts of the bacteria are purified by silica gel chromatography and HPLC. The purified and crude extracts were applied to leguminous plants to demonstrate their effectiveness.

In order to facilitate a better understanding of the present invention, the following examples are given primarily for the purposes of illustrating certain more specific details thereof.

PROCEDURE I

PURIFICATION OF THE PHYTOHORMONE:

A. Preparation of soybean seed extracts (SSE)

Soybean seeds (*Glycine max* cv. Essex) were rinsed with distilled water, soaked in ethanol:water mix (50:50 V/V; 1 mL per seed) and placed in a shaker incubator at 30° C. overnight. The seed extracts were then cleared by filtration through a 6 mm pore filter (Millipore) and tenfold concentrated by rotary evaporation. The concentrated soybean seed extracts (SSE) were sterilized by filtration through a 0.25 μm pore filter (Millipore) and kept frozen at −20° C. until use.

B. Preparation of *B. japonicum* extracts

Cells of *B. japonicum.* strains USDA110 or USDA135, were grown in 500 mL of minimal medium (Bergensen's MM) with glycerol as carbon source to late stationary phase ($O.D._{600}$ of about 1.0) in a shaker incubator at 30° C. This culture was used to inoculate 5×1 L of minimal medium of 4 L flasks to an $O.D._{600}$ from about 0.05 to about 0.08. The cultures were grown for 4–6 hours with shaking at 30° C. The SSE was diluted with distilled water to a concentration wherein 1 L of solution contained the extract of 100 soybean seeds. The diluted SSE was added (10 mL of SSE per L of culture) to the culture. Incubation was continued for 40 hours. Alternatively, the phytohormone was induced in the *B. japonicum* culture by the addition of genistein (preferably, at a concentration of 2 μm). Genistein, daidzein and their glycosylated derivatives have been shown to be active nod gene inducing flavonoids in SSE. The entire culture, or alternatively the supernatant, was extracted with 0.3 volumes of n-butanol (Mallinckrodt, n-butyl alcohol, nanograde) by shaking at 30° C. for 3 hours. The butanol and water phases were allowed to separate by standing overnight at room temperature. The butanol was then collected and the extract concentrated to dryness by rotary evaporation. The dried extract containing the phytohormone was resuspended in acetonitrile:water (50:50 V/V) and stored at room temperature until use.

C. Silica gel chromatography purification

A chromatography column (Pharmacia, C-column, 1.6×100 cm) was filled with silica gel with a particle size between 0.63 and 0.2 mm (EM Science, Silica Gel 60) prepared with acetonitrile:water (60:40 V/V). The column was washed successively with 200 mL of 82% acetonitrile, 60% acetonitrile and 82% acetonitrile. The sample was prepared by bringing the acetonitrile from 50% to 82% and then running the sample through the column. When all of the samples had been entered on the column, the column was washed with 500 mL of 82% acetonitrile. The sample was eluted in 60% acetonitrile at a flow rate of about 1.5 mL per minute. Fractions were collected and further purified by HPLC.

D. HPLC purification

The fractions from the silica gel purification were applied to a binary solvent HPLC system (two Waters MODEL 501 HPLC pumps controlled by a Waters BASELINE 810 chromatography workstation with an installed computer, Model APC IV by NEC; a 4×250 mm HPLC column, Pharmacia-LKB PEP-S C2/C18, with a guard column; detection at 206 nm with a tunable absorbance detector, Waters MODEL 484). The phytohormone was purified by four successive passes through the HPLC system. For the first pass, the HPLC system was programmed to supply 20% acetonitrile in water for the first 5 minutes, go to 40% acetonitrile in water in 10 minutes, remain at 40% acetonitrile in water for 25 minutes, and then go to 60% acetonitrile in water in 5 minutes. The total duration of the program was 45 minutes with the phytohormone eluting at about 30 minutes. For the second pass, the HPLC system was programmed to supply 20% acetonitrile in water for the first 5 minutes, go to 42% acetonitrile in water in 5 minutes, remain at 42% acetonitrile in water for 20 minutes, and then go to 60% acetonitrile in water in 5 minutes. The total duration of the program was 35 minutes with the phytohormone eluting at about 21 minutes. For the third pass, the HPLC system was programmed to supply 20% acetonitrile in water for the first 5 minutes, go to 45 acetonitrile in water in 5 minutes, remain at 46% acetonitrile in water for 20 minutes, and then go to 60% acetonitrile in water in 5 minutes. The total duration of the program was 35 minutes with the phytohormone eluting at between about 17 and about 18 minutes. For the fourth pass, the HPLC system was again programmed to supply 20% acetonitrile in water for the first 5 minutes, go to 40% acetonitrile in water in 25 minutes, remain at 40% acetonitrile in water for 20 minutes, and then go to 60% acetonitrile in water in 5 minutes. The total duration of the program was 45 minutes with the phytohormone eluting at about 30 minutes. The total yield is about 0.2 to 0.3 mg of phytohormone per liter of induced culture. The structure of the phytohormone was determined and verified by nuclear magnetic resonance and mass spectroscopy. BJ Nod-V (C18:1) was derived from strain USDA110. BJ Nod-V (Ac, C18:1), BJ Nod-V (C16:0), BJ Nod-V (Ac, C16:0) and BJ Nod-V (C16:1) were derived from strain USDA135.

PROCEDURE II

Treatment of Legumes with the Phytohormone

Legumes were treated with the phytohormone in its crude state (i.e., the butanol extract from Procedure I,B hereinabove) and in its purified state (i.e., the phytohormone from Procedure I,D hereinabove). The phytohormone was effective at inducing several biological activities.

A. The induction of root hair curling

Dilutions from 10^{-3} to 10^{-2} of the phytohormone were added to hydroponically grown legume seedlings (soybean, *Glycine soja,* and siratro, *Macroptilium atropurpureum*). The root hairs of the seedlings were examined 24–96 hours after addition of the phytohormone. Parallel controls were run in which similar amounts of solvent, without the phytohormone, were

added, as well as plants with no additions. Biological activity of the phytohormone were scored on a scale of 1 to 5 with 5 exhibiting marked deformations and swelling of the root hairs. Phytohormone activity was detectable, a grade of 3 or higher on the scale, down to a concentration of at least 10^{-12} M At concentrations of greater than about 10^{-3}M, the phytohormone exhibited an inhibitory effect with respect to root hair curling.

B. The induction of flavonoids

Dilutions from 10^{-3} M to 10^{-12} M of the phytohormone were added to soybean seedlings grown hydroponically. Samples of the growth medium were removed between 4 and 12 days after addition of the phytohormone. The medium samples were then tested for their ability to induce a nodY-lacZ fusion in *B. japonicum*, strain USDA135. The phytohormone induced the production of flavonoids in the treated plants. High levels of the phytohormone inhibit flavonoid production.

C. The induction of cortical cell division

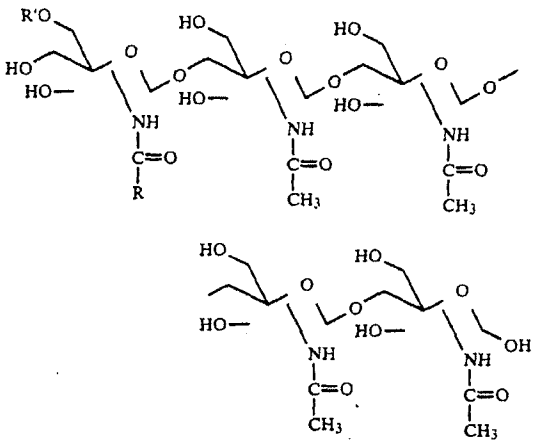
At the end of the incubation period for each of the above tests, the plants were removed and the roots were sectioned by hand. The roots were examined for the presence of foci of root cell division in accordance with N. Deshmane and G. Stacey, *J. of Bacteriology*, 171 (1989), pp. 3324–3330. Small nodule-like structures equivalent in number and size to foci on like roots grown in a like medium for a like time period, formed on the treated roots within 9–12 days of the addition of the phytohormone. This is a similar time scale as the formation of bacterium-induced nodulation. The activity of the phytohormone was detectable down to a concentration of at least 10^{-} M. At concentrations of greater than about 10^{-3} M, the phytohormone exhibited an inhibitory effect on the plants with respect to cortical cell division.

Thus, the present invention provides a phytohormone for inducing root hair curling and nodulation in leguminous plants. This induction is accomplished in the absence of nitrogen-fixing bacteria.

Various of the features of the invention which are believed to be new are set forth in the appended claims.

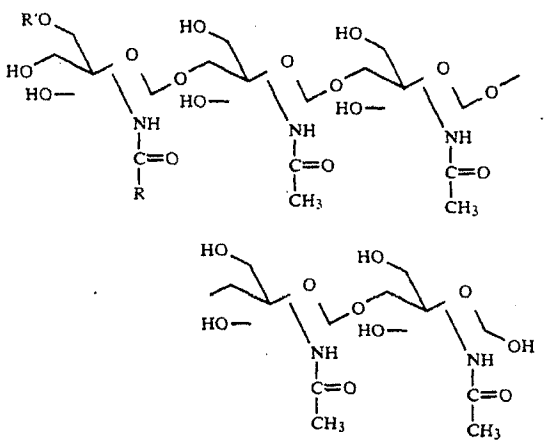
What is claimed is:

1. A phytohormone for inducing root hair curling and root nodulation in the roots of leguminous plants, wherein said hormone is a pentasaccharide having the structure:



wherein R is selected from the group consisting of $(CH_2)_{15}CH_3$ and $(CH_2)_n—CH=CH—(CH_2)_m—CH_3$, wherein n+m is 13 or 15 and R′ is selected from the group consisting of H and $CH_3CH_2C=0$.

2. A method for treating the roots of leguminous plants for inducing root hair curling and root nodulation, said method comprising treating the roots of the plants with a pentasaccharide having the structure:



wherein R is selected from the group $(CH_2)_{15}CH_3$ and $(CH_2)_n—CH=CH—(CH_2)_m—CH_3$, wherein n+m is 13 or 15 and R′ is selected from the group consisting of H and $CH_3CH_2C=0$.

3. The method of claim 2 wherein the roots of the plants are treated with said phytohormone at a concentration of from about 10^{-12} M to about 10^{-3} M.

4. The method of claim 2 wherein said roots are treated with said phytohormone in the absence of nitrogen-fixing bacteria.

5. The method of claim 4 wherein said treated roots exhibit foci of root cell division equivalent to like roots grown in the presence of nitrogen-fixing bacteria but without the addition of said phytohormone.

6. The method of claim 2 wherein said phytohormone induces the production of flavonoids by said plants.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,175,149

DATED : December 29, 1992

INVENTOR(S) : Gary Stacey, Russell W. Carlson & Herman Spaink

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page: Item [57]

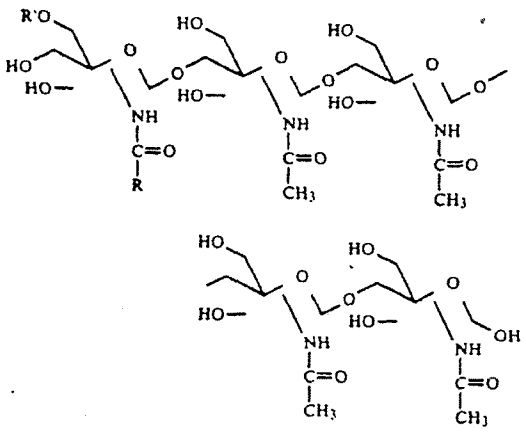
Abstract:

At line 4, delete "pentassaccharide" and add -- pentasaccharide --.

line 5, delete "a" and add --A --.

At column 2, line 26, before "the", add -- In --.

At column 2, lines 6-25, delete:



CERTIFICATE OF CORRECTION

PATENT NO. : 5,175,149 Page 2 of 5
DATED : December 29, 1992
INVENTOR(S) : Gary Stacey, Russell W. Carlson & Herman Spaink

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

and insert

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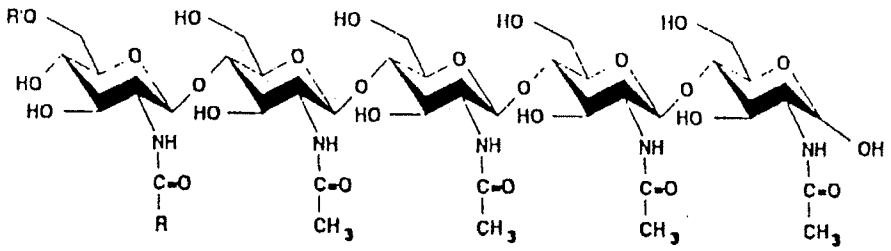

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At column 4, line 61, delete $_{10}-^{2}$ and add -- $_{10}-^{12}$ --.

At column 5, line 6, after "M", insert -- . --.

At column 5, line 46, after "10_" and insert $_{10}-^{12}$ --

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

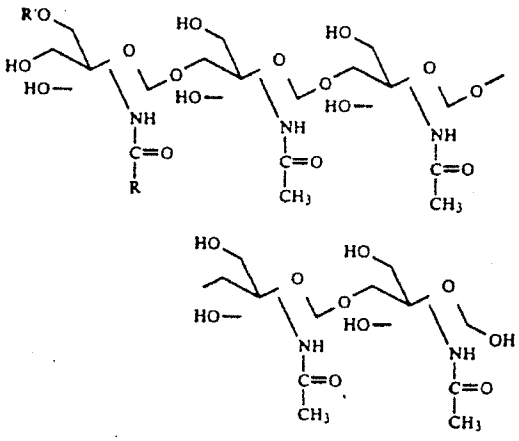
PATENT NO. : 5,175,149

DATED : December 29, 1992

INVENTOR(S) : Gary Stacey, Russell W. Carlson & Herman Spaink

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

At column 6, lines 1-20, delete



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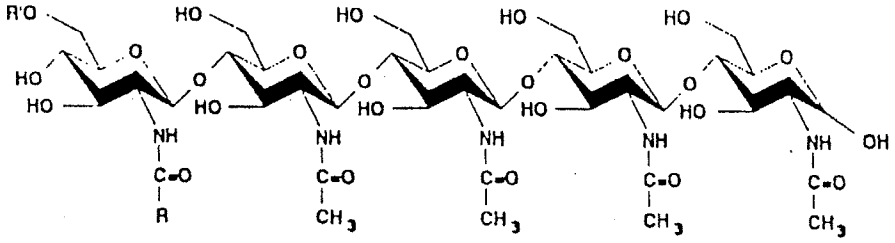

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UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

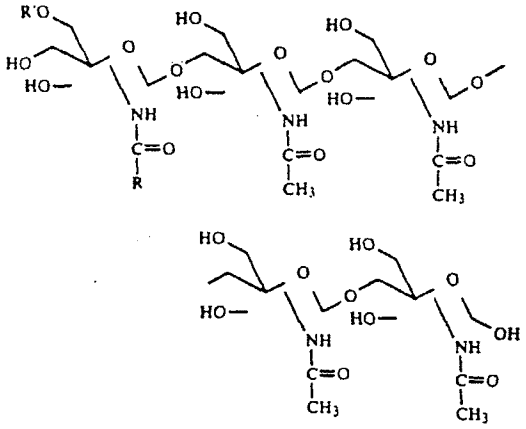
PATENT NO. : 5,175,149

DATED : December 29, 1992

INVENTOR(S) : Gary Stacey, Russell W. Carlson & Herman Spaink

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

At column 6, lines 29-48, delete



UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,175,149 Page 5 of 5

DATED : December 29, 1992

INVENTOR(S) : Gary Stacey, Russell W. Carlson & Herman Spaink

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

and insert

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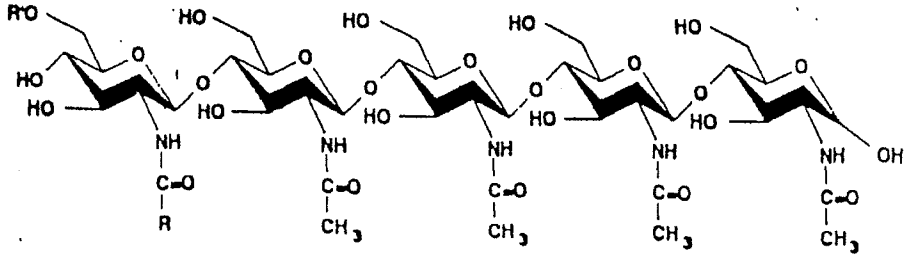

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Signed and Sealed this

Fifth Day of July, 1994

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

EXHIBIT E

US005321011A

United States Patent [19]

Stacey et al.

[11] Patent Number: 5,321,011

[45] Date of Patent: * Jun. 14, 1994

[54] **PENTASACCHARIDE PHYTOHORMONES AND METHODS FOR THEIR USE**

[75] Inventors: **Gary Stacey**, Knoxville, Tenn.; **Russell W. Carlson**, Athens, Ga.; **Herman Spaink**, Leiden, Netherlands

[73] Assignee: **The University of Tennessee Research Corporation**, Knoxville, Tenn.

[*] Notice: The portion of the term of this patent subsequent to Dec. 29, 2009 has been disclaimed.

[21] Appl. No.: **822,925**

[22] Filed: **Jan. 21, 1992**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 771,356, Oct. 4, 1991, Pat. No. 5,175,149.

[51] **Int. Cl.**[5] **A61K 31/70**; C07H 5/06; C07H 13/06

[52] **U.S. Cl.** **514/23**; 514/25; 514/54; 514/55; 514/62; 536/53; 536/55.1; 536/55.2; 71/7

[58] **Field of Search** 536/53, 55.1, 55.2; 47/57.6; 514/23, 25, 54, 55, 62; 71/7

[56] **References Cited**

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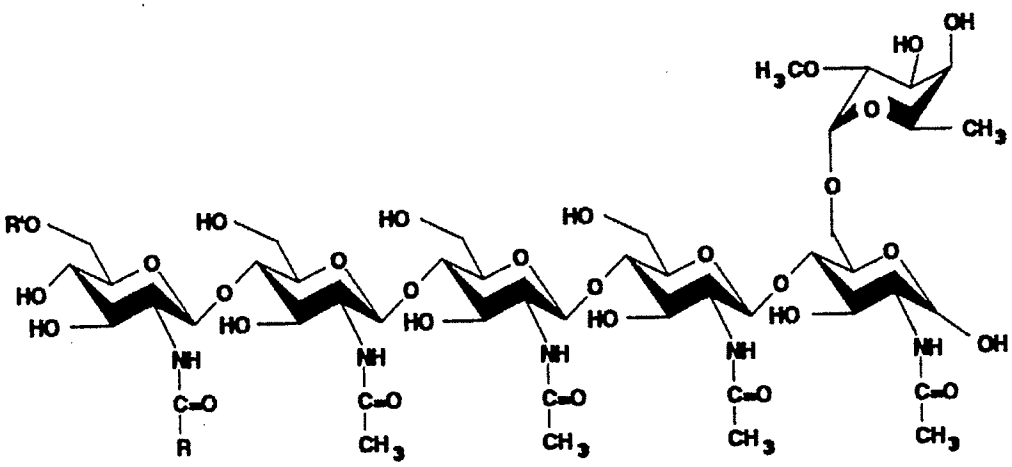
Dazzo, F. B. et al., "*Rhizobium* Lipopolysaccharide Modulates Infection Thread Development in White Clover Root Hairs" *J. Bacteriology*, v. 173, No. 17, pp. 5371–5384, 1991.

Primary Examiner—Johnnie R. Brown
Assistant Examiner—Anita Varma
Attorney, Agent, or Firm—Luedeka, Neely & Graham

[57] **ABSTRACT**

A phytohormone for inducing root hair curling and root nodulation in the roots of leguminous plants particularly in the absence of nitrogen-fixing bacteria. The phytohormone comprises a pentasaccharide having a fatty acid condensed on the non-reducing end. A method for treating the roots of leguminous plants for inducing root hair curling and root nodulation is also disclosed.

6 Claims, 1 Drawing Sheet



Compound	R	R'	m + n
BJ Nod-V (C18:1)	$(CH_2)_7-CH=CH-(CH_2)_7CH_3$	H	–
BJ Nod-V (Ac, C18:1)	$(CH_2)_7-CH=CH-(CH_2)_7CH_3$	$CH_3CH_2C(=O)$	–
BJ Nod-V (C16:0)	$(CH_2)_{15}CH_3$	H	–
BJ Nod-V (Ac, C16:0)	$(CH_2)_{15}CH_3$	$CH_3CH_2C(=O)$	–
BJ Nod-V (C16:1)	$(CH_2)_5-CH=CH-(CH_2)_5-CH_3$	H	13

Fig. 1

PENTASACCHARIDE PHYTOHORMONES AND METHODS FOR THEIR USE

This is a continuation-in-part of application Ser. No. 07/771,356, filed Oct. 4, 1991, now U.S. Pat. No. 5,175,149, issued Dec. 29, 1992.

The present invention relates to compounds and compositions for inducing changes in plants. The present invention also relates to methods for the application of those compounds and compositions to plants.

Phytohormones are compounds which induce changes in plants. Such compounds are useful in the communication between plants and other organisms. For example, many species of plants produce a phytohormone which is released from their roots into the soil. The hormone prevents the roots of plants of the same species from growing in that area. Therefore, plants of the same species in a limited area will have root systems that do not overlap.

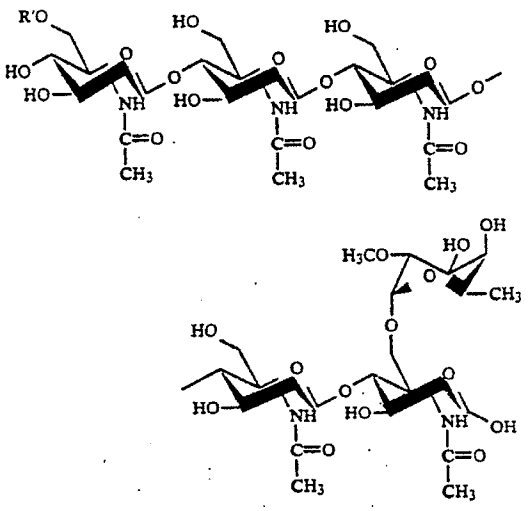
Phytohormones are also released by organisms other than plants. Nitrogen-fixing bacteria, such as Rhizobium, Bradyrhizobium, and Azorhizobium species, release hormones in the vicinity of the roots of leguminous plants, such as soybean or alfalfa. Such hormones induce nodulation and root hair curling in the plants which make the plant roots suitable for symbiotic inhabitation by the bacteria. The bacteria and the plants then exist in a symbiotic relationship with the bacteria fixing nitrogen into the soil and the plants providing food to the bacteria.

Generally, the nitrogen-fixing bacteria produce the root hair curling and root nodulation phytohormone in response to the production of flavonoids by the plants. Flavonoids comprise a large group of secondary products found in all higher plants. It is believed that flavonoids induce the expression of genes in the bacteria necessary to produce the phytohormone. The proteins believed to be involved in phytohormone production are encoded by the nod and nol genes. The phytohormone is then secreted and the plants respond with the production of nodules and curled root hairs.

In order to promote the effective inoculation of leguminous plants with specific strains of rhizobial bacteria, those strains are presently coated on the seeds or introduced into the soil of the plants. Soybean farmers sow seeds which are coated with those rhizobial strains which are known to be effective nitrogen-fixers. The soil may also be pretreated with the bacteria prior to sowing. In either case, the soybean seedlings rely solely on the ability of the particular strain of bacterium to induce root hair curling and root nodulation.

Although this process is more effective than natural processes for the inoculation of the plant, the rhizobial bacteria will often be insufficient to induce the desired level of nodulation. The desired particular strains of bacteria may be partially or totally supplanted by the natural rhizobial species in the soil or the desired strains may not be particularly efficient at inducing the production of nodules for the bacteria to inhabit. The mere coating of the leguminous seeds or sowing of the soil with the desired bacterial strains does not necessarily lead to the desired inoculation of the plant. Therefore, it is desirable to have a means for inducing nodulation on the roots of leguminous plants that is independent of the presence of rhizobial bacteria.

It has not been possible previously to induce root hair curling and root nodulation in leguminous plants in the absence of nitrogen-fixing bacteria. The present inventors have discovered and purified a phytohormone which is capable of inducing root hair curling and root nodulation in the roots of leguminous plants. The phytohormone is a pentasaccharide of N-acetylglucosamine having a fatty acid condensed on the non-reducing end. The phytohormone of the present invention has the structure:



In the pentasaccharide, R is a fatty acid condensate and R′ is selected from the group consisting of H and an acetyl, $CH_3CH_2C{=}O$. The fatty acid is selected from the group consisting of $(CH_2)_{15}{-}CH_3$, $(CH_2)_8{-}CH{=}CH{-}(CH_2)_7{-}CH_3$, and $(CH_2)_n{-}CH{=}CH{-}(CH_2)_m{-}CH_3$, where n+m is 13. These fatty acids are alternatively designated using the terms C16:0, C18:1 and C16:1.

In accordance with the method of the present invention root hair curling and root nodulation of the roots of leguminous plants are induced. The method comprises treating the roots of the plant with a pentasaccharide phytohormone having a fatty acid condensed on the non-reducing end. The pentasaccharide has the structure shown herein above and is applied to the plant root in a concentration of from about 10^{-12}M to about 10^{-3}M. At concentrations greater than about 10^{-3}M the hormone actually inhibits root hair curling and root nodulation. Thus, care must be taken when treating the roots of the selected leguminous plant with the phytohormone.

A drawing of the phytohormone is shown in FIG. 1. The depicted phytohormone is a pentasaccharide of N-acetylglucosamine. The non-reducing end of the pentasaccharide is substituted with a C18:1, C16:1, or C16:0 fatty acid. Further, the non-reducing sugar may be acetylated. The reducing end of the pentasaccharide is substituted with an α-2-0-methylfucose moiety at the 6-position of the reducing sugar.

The phytohormone is named as follows: "BJ" indicates that the compound is isolated from *Bradyrhizobium japonicum;* "Nod" indicates that the compound is involved in nodulation; "−V" indicates that there are five N-acetylglucosamines in the compound structure. The terms in the parentheses indicate whether the compound is acetylated ("Ac") or not at the R′ position and the structure of the fatty acid at the

R position. The term C18:1 indicates an 18 carbon chain with one double bond; C16:0 indicates a 16 carbon chain with no double bond; and C16:1 indicates a 16 carbon chain with one double bond. Thus, the phytohormone BJ Nod-V (Ac, C18:1) is a nodulating pentasaccharide derived from *B. japonicum* which has been acetylated and contains an 18 carbon fatty acid residue with one double bond.

The phytohormone preferably is purified by recovery from *Bradyrhizobium japonicum* which has been induced to produce the phytohormone by the presence of flavonoids. The crude extracts of the bacteria are purified by silica gel chromatography and HPLC. The purified and crude extracts were applied to leguminous plants to demonstrate their effectiveness.

In order to facilitate a better understanding of the present invention, the following examples are given primarily for the purposes of illustrating certain more specific details thereof.

PROCEDURE I

Purification of the Phytohormone

A. Preparation of soybean seed extracts (SSE): Soybean seeds (*Glycine max* cv. Essex) were rinsed with distilled water, soaked in ethanol:water mix (50:50 V/V; 1 mL per seed) and placed in a shaker incubator at 30° C. overnight. The seed extracts were then cleared by filtration through a 6 mm pore filter (Millipore) and tenfold concentrated by rotary evaporation. The concentrated soybean seed extracts (SSE) were sterilized by filtration through a 0.25 μm pore filter (Millipore) and kept frozen at −20° C. until use.

B. Preparation of *B. japonicum* extracts: Cells of *B. japonicum*, strains USDA110 or USDA135, were grown in 500 mL of minimal medium (Bergensen's MM) with glycerol as carbon source to late stationary phase ($O.D._{600}$ of about 1.0) in a shaker incubator at 30° C. This culture was used to inoculate 5×1 L of minimal medium of 4 L flasks to an $O.D._{600}$ from about 0.05 to about 0.08. The cultures were grown for 4–6 hours with shaking at 30° C. The SSE was diluted with distilled water to a concentration wherein 1 L of solution contained the extract of 100 soybean seeds. The diluted SSE was added (10 mL of SSE per L of culture) to the culture. Incubation was continued for 40 hours. Alternatively, the phytohormone was induced in the *B. japonicum* culture by the addition of genistein (preferably, at a concentration of 2 μm). Genistein, daidzein and their glycosylated derivatives have been shown to be active nod gene inducing flavonoids in SSE. The entire culture, or alternatively the supernatant, was extracted with 0.3 volumes of n-butanol (Mallinckrodt, n-butyl alcohol, nanograde) by shaking at 30° C. for 3 hours. The butanol and water phases were allowed to separate by standing overnight at room temperature. The butanol was then collected and the extract concentrated to dryness by rotary evaporation. The dried extract containing the phytohormone was resuspended in acetonitrile:water (50:50 V/V) and stored at room temperature until use.

C. Silica gel chromatography purification: A chromatography column (Pharmacia, C-column, 1.6×100 cm) was filled with silica gel with a particle size between 0.63 and 0.2 mm (EM Science, Silica Gel 60) prepared with acetonitrile:water (60:40 V/V). The column was washed successively with 200 mL of 82% acetonitrile, 60% acetonitrile and 82% acetonitrile. The sample was prepared by bringing the acetonitrile from 50% to 82% and then running the sample through the column. When all of the samples had been entered on the column, the column was washed with 500 mL of 82% acetonitrile. The sample was eluted in 60% acetonitrile at a flow rate of about 1.5 mL per minute. Fractions were collected and further purified by HPLC.

D. HPLC purification: The fractions from the silica gel purification were applied to a binary solvent HPLC system (two Waters MODEL 501 HPLC pumps controlled by a Waters BASELINE 810 chromatography workstation with an installed computer, Model APC IV by NEC; a 4×250 mm HPLC column, Pharmacia-LKB PEP-S C2/C18, with a guard column; detection at 206 nm with a tunable absorbance detector, Waters MODEL 484). The phytohormone was purified by four successive passes through the HPLC system. For the first pass, the HPLC system was programmed to supply 20% acetonitrile in water for the first 5 minutes, go to 40% acetonitrile in water in 10 minutes, remain at 40% acetonitrile in water for 25 minutes, and then go to 60% acetonitrile in water in 5 minutes. The total duration of the program was 45 minutes with the phytohormone eluting at about 30 minutes. For the second pass, the HPLC system was programmed to supply 20% acetonitrile in water for the first 5 minutes, go to 42% acetonitrile in water in 5 minutes, remain at 42% acetonitrile in water for 20 minutes, and then go to 60% acetonitrile in water in 5 minutes. The total duration of the program was 35 minutes with the phytohormone eluting at about 21 minutes. For the third pass, the HPLC system was programmed to supply 20% acetonitrile in water for the first 5 minutes, go to 45 acetonitrile in water in 5 minutes, remain at 46% acetonitrile in water for 20 minutes, and then go to 60% acetonitrile in water in 5 minutes. The total duration of the program was 35 minutes with the phytohormone eluting at between about 17 and about 18 minutes. For the fourth pass, the HPLC system was again programmed to supply 20% acetonitrile in water for the first 5 minutes, go to 40% acetonitrile in water in 25 minutes, remain at 40% acetonitrile in water for 20 minutes, and then go to 60% acetonitrile in water in 5 minutes. The total duration of the program was 45 minutes with the phytohormone eluting at about 30 minutes. The total yield is about 0.2 to 0.3 mg of phytohormone per liter of induced culture. The structure of the phytohormone was determined and verified by nuclear magnetic resonance and mass spectroscopy. BJ Nod-V (C18:1) was derived from strain USDAI10. BJ Nod-V (Ac, C18:1), BJ Nod-V (C16:0), BJ Nod-V (Ac, C16:0) and BJ Nod-V (C16:1) were derived from strain USDA135.

PROCEDURE II

Treatment of Legumes with the Phytohormone

Legumes were treated with the phytohormone in its crude state (i.e., the butanol extract from Procedure I,B hereinabove) and in its purified state (i.e., the phytohormone from Procedure I,D hereinabove). The phytohormone was effective at inducing several biological activities.

A. The induction of root hair curling: Dilutions from 10^{-3}M to 10^{-12} of the phytohormone were added to hydroponically grown legume seedlings (soybean, *Glycine soja*, and siratro, *Macroptilium atropurpureum*). The root hairs of the seedlings were examined 24–96 hours after addition of the phytohormone. Parallel controls were run in which similar amounts of solvent, without

the phytohormone, were added, as well as plants with no additions. Biological activity of the phytohormone were scored on a scale of 1 to 5 with 5 exhibiting marked deformations and swelling of the root hairs. Phytohormone activity was detectable, a grade of 3 or higher on the scale, down to a concentration of at least 10^{-12}M. At concentrations of greater than about 10^{-3}M, the phytohormone exhibited an inhibitory effect with respect to root hair curling.

B. The induction of flavonoids: Dilutions from 10^{-3}M to 10^{-12}M of the phytohormone were added to soybean seedlings grown hydroponically. Samples of the growth medium were removed between 4 and 12 days after addition of the phytohormone. The medium samples were then tested for their ability to induce a nodY-lacZ fusion in *B. japonicum*, strain USDA135. The phytohormone induced the production of flavonoids in the treated plants. High levels of the phytohormone inhibit flavonoid production.

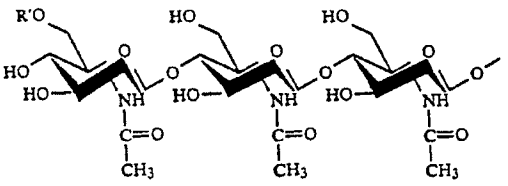
C. The induction of cortical cell division: At the end of the incubation period for each of the above tests, the plants were removed and the roots were sectioned by hand. The roots were examined for the presence of foci of root cell division in accordance with N. Deshmane and G. Stacey, *J. of Bacteriology*, 171 (1989), pp. 3324–3330. Small nodule-like structures equivalent in number and size to foci on like roots grown in a like medium for a like time period, formed on the treated roots within 9–12 days of the addition of the phytohormone. This is a similar time scale as the formation of bacterium-induced nodulation. The activity of the phytohormone was detectable down to a concentration of at least 10^{-12}M. At concentrations of greater than about 10^{-3}M, the phytohormone exhibited an inhibitory effect on the plants with respect to cortical cell division.

Thus, the present invention provides a phytohormone for inducing root hair curling and nodulation in leguminous plants. This induction is accomplished in the absence of nitrogen-fixing bacteria.

Various of the features of the invention which are believed to be new are set forth in the appended claims.

What is claimed is:

1. A phytohormone for inducing root hair curling and root nodulation in the roots of leguminous plants, said phytohormone is a 6-α-2-O-methylfucose pentasaccharide having the structure:



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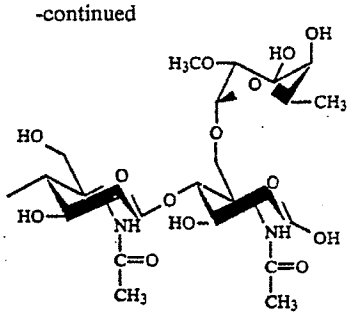
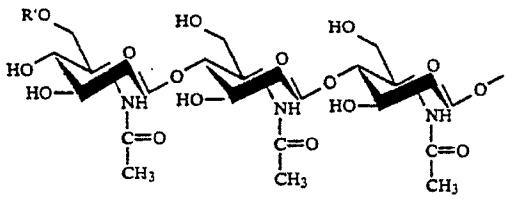
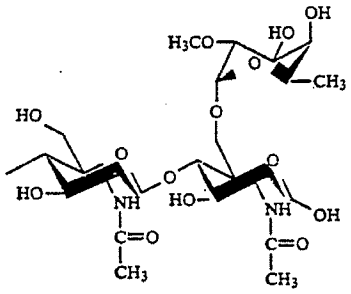


wherein R is selected from the group consisting of $(CH_2)_{15}CH_3$ and $(CH_2)_n—CH=CH—(CH_2)_m—CH_3$, wherein n+m is 13 or 15 and R′ is selected from the group consisting of H and $CH_3CH_2C=O$.

2. A method for treating the roots of leguminous plants for inducing root hair curling and root nodulation, said method comprising treating the roots of the plants with a 6-α-2-O-methylfucose pentasaccharide having the structure:





wherein R is selected from the group consisting of $(CH_2)_{15}CH_3$ and $(CH_2)_n—CH=CH—(CH_2)_m—CH_3$, wherein n+m is 13 or 15 and R′ is selected from the group consisting of H and $CH_3CH_2C=O$.

3. The method of claim 2 wherein the roots of the plants are treated with said 6-α-2-O-methylfucose pentasaccharide at a concentration of from about 10^{-12}M to about 10^{-3}M.

4. The method of claim 2 wherein said roots are treated with said 6-α-2-O-methylfucose pentasaccharide in the absence of nitrogen fixing bacteria.

5. The method of claim 4 wherein said treated roots exhibit foci of root cell division equivalent to roots of like plants grown in the presence of nitrogen-fixing bacteria but without the addition of said 6-α-2-O-methylfucose pentasaccharide to said roots of said like plants.

6. The method of claim 2 wherein said 6-α-2-O-methylfucose pentasaccharide induces the production of flavonoids by said plants.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,321,011

DATED : June 14, 1994

INVENTOR(S) : Gary Stacey, Russell W. Carleson, Herman Spaink

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 4, Line 48

Delete "USDAI10" and insert --USDA 110--.

Signed and Sealed this

First Day of November, 1994

Attest:

Attesting Officer

BRUCE LEHMAN

Commissioner of Patents and Trademarks

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO : 5,321,011

DATED : June 14, 1994

INVENTOR(S) : Stacey et al.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 2, lines 10-30, in the phytohormone structure, delete the terminal "CH_3" group located at the end of the bond line leading from the "C=O" connected through the "NH" to the first or leftmost acetylyglucosamine unit and replace the "CH_3" group at that site with --R-- so that the resulting phytohormone structure corresponds to the phytohormone structure of Fig. 1.

Column 4, line 32, after "45" and before "acetonitrile", insert "%".

Column 5, line 59 - column 6, line 13, in the phytohormone structure, delete the terminal "CH_3" group located at the end of the bond line leading from the "C=O" connected through the "NH" to the first or leftmost acetylyglucosamine unit and replace the "CH_3" group at that site with --R-- so that the resulting phytohormone structure corresponds to the phytohormone structure of Fig. 1.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO : 5,321,011
DATED : April 15, 1999
INVENTOR(S) : Stacey et al.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 6, lines 25-45, in the phytohormone structure, delete the terminal "CH_3" group located at the end of the bond line leading from the "C=0" connected through the "NH" to the first or leftmost acetylyglucosamine unit and replace the "CH_3" group at that site with --R-- so that the resulting phytohormone structure corresponds to the phytohormone structure of Fig. 1.

Signed and Sealed this

Fourteenth Day of December, 1999

Attest:

Q. TODD DICKINSON

Attesting Officer *Acting Commissioner of Patents and Trademarks*

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

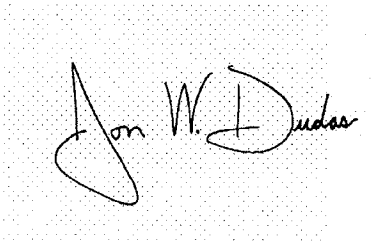
PATENT NO. : 5,321,011
APPLICATION NO. : 07/822925
DATED : June 14, 1994
INVENTOR(S) : Gary Stacey et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title page, (*) notice the Notice should read as follows:
-- [*] Notice: The portion of the term of this patent subsequent to Oct. 4, 2011 has been disclaimed. --

Signed and Sealed this

Seventh Day of November, 2006



JON W. DUDAS
Director of the United States Patent and Trademark Office

EXHIBIT F

US007262151B2

(12) **United States Patent**
Smith et al.

(10) **Patent No.: US 7,262,151 B2**
(45) **Date of Patent: Aug. 28, 2007**

(54) **METHODS AND COMPOSITIONS FOR PRODUCTION OF LIPO-CHITO OLIGOSACCHARIDES BY RHIZOBACTERIA**

(75) Inventors: **Donald L. Smith**, Ste-Anne-de-Bellevue (CA); **Fazli Mabood**, Ste-Anne-de-Bellevue (CA); **Hao Zhang**, San Diego (CA)

(73) Assignee: **McGill University**, Montreal, Quebec (CA)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/146,034**

(22) Filed: **May 16, 2002**

(65) **Prior Publication Data**

US 2003/0096375 A1 May 22, 2003

Related U.S. Application Data

(60) Provisional application No. 60/303,037, filed on Jul. 6, 2001.

(51) **Int. Cl.**
A01N 63/00 (2006.01)
C12P 19/44 (2006.01)

(52) **U.S. Cl.** **504/117**; 435/252.2; 424/93.4

(58) **Field of Classification Search** 435/74, 435/72, 84, 252.2; 424/93.4; 504/117
See application file for complete search history.

(56) **References Cited**

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Mabood, Fazli, Jasmonates induce the expressoin of *nod* genes in *Bradyrhizobium japonicum*, May 17, 2001.
Mabood, Fazli, Linoleic and linolenic acid induce the expression of *nod* genes in *Bradyrhizobium japonicum* USDA 3, May 17, 2001.

* cited by examiner

Primary Examiner—Leon B. Lankford, Jr.
Assistant Examiner—Susan Hanley

(57) **ABSTRACT**

Lipo-chito oligosaccharides (LCOs) are produced by culturing rhizobacteria cells in or on a culture medium comprising at least one of: jasmonic acid or a derivative thereof; linoleic acid or a derivative thereof; or linolenic acid or a derivative thereof. Preferably, the rhizobacteria cells are *Bradyrhizobium japonicum* cells having the identifying characteristics of *B. japonicum* strain USDA 3. Preferably, the derivative of jasmonic acid is an ester thereof, preferably methyl jasmonate. Also provided are methods for improving LCO production at low temperatures, particularly temperatures below 25° C.

20 Claims, 6 Drawing Sheets

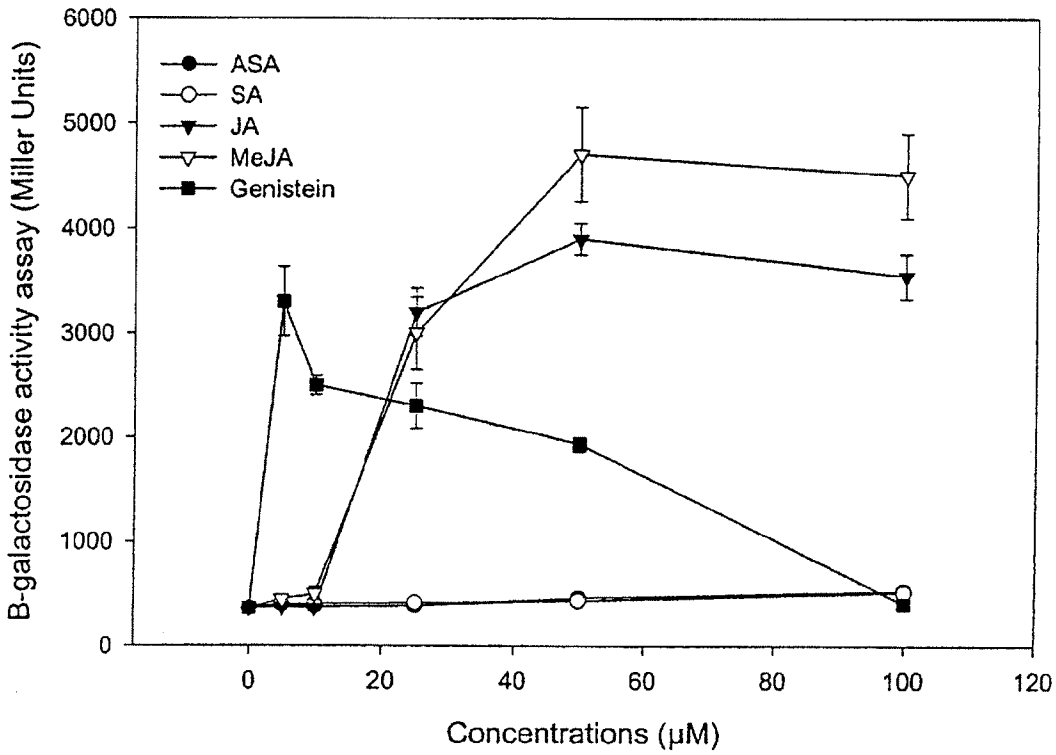

6000
5000
4000
3000
2000
1000
0
B-galactosidase activity assay (Miller Units)
ASA
SA
JA
MeJA
Genistein
0
20
40
60
80
100
120
Concentrations (μM)

Fig. 1

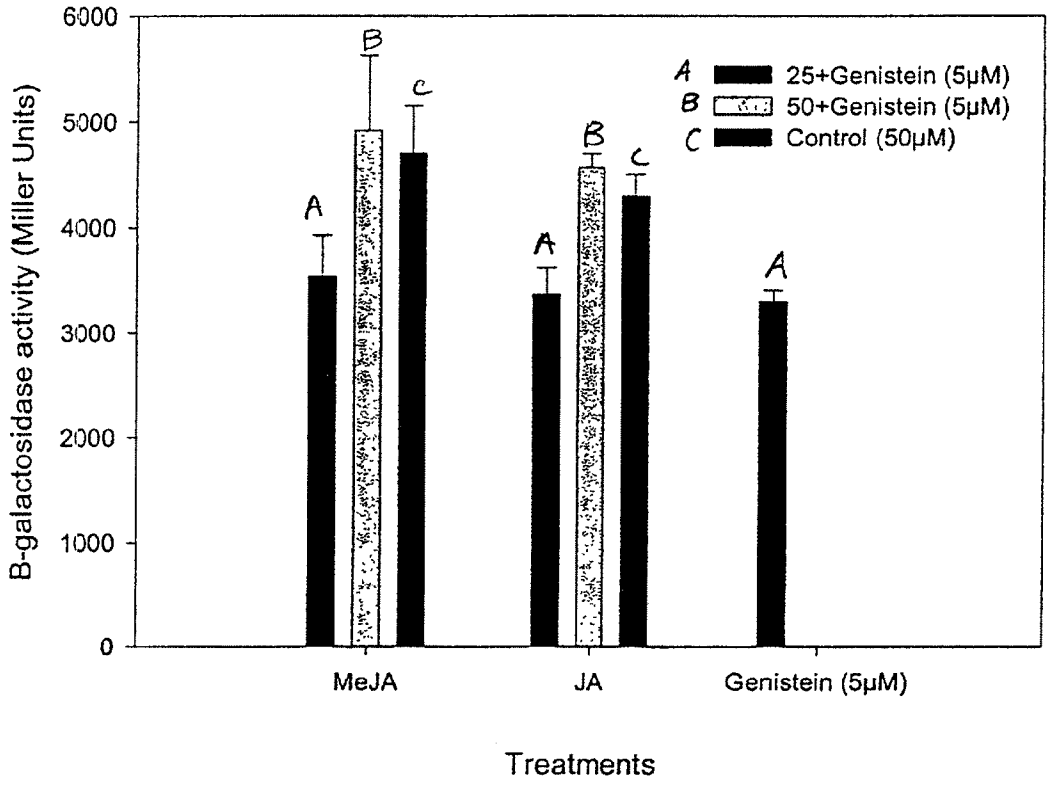

6000
5000
4000
3000
2000
1000
0
B-galactosidase activity (Miller Units)
A
B
C
25+Genistein (5μM)
50+Genistein (5μM)
Control (50μM)
MeJA
JA
Genistein (5μM)
Treatments

Fig. 2

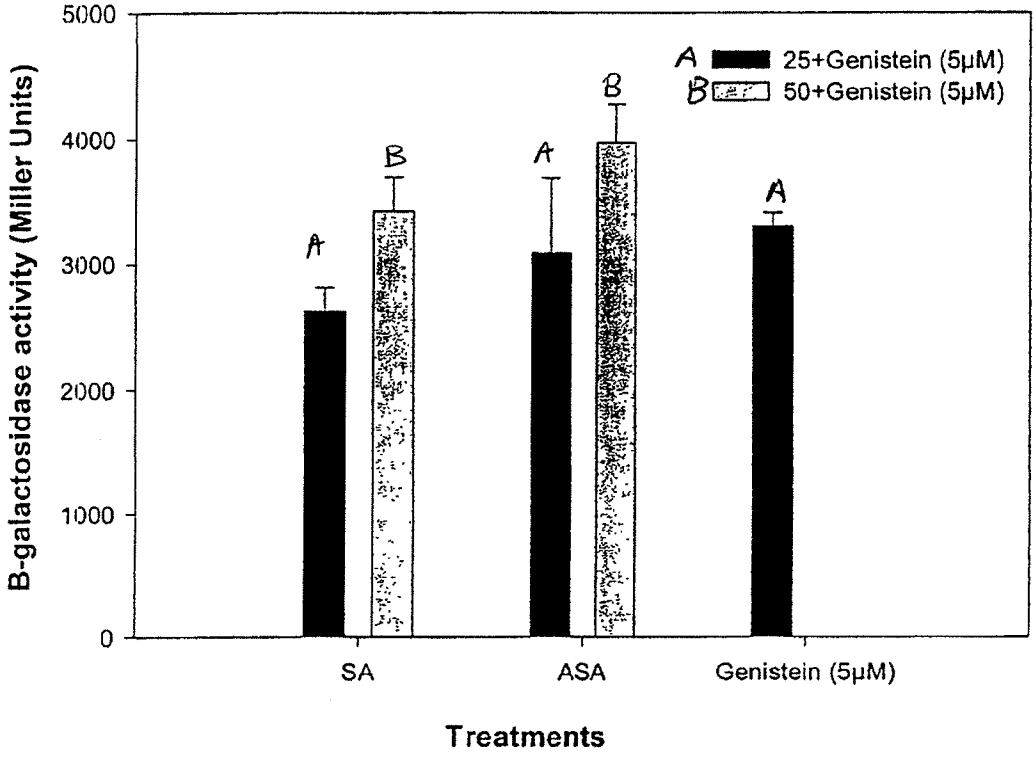

5000
4000
3000
2000
1000
0
B-galactosidase activity (Miller Units)
A
B
A
B
A
B
A 25+Genistein (5μM)
B 50+Genistein (5μM)
SA
ASA
Genistein (5μM)
Treatments

Fig. 3

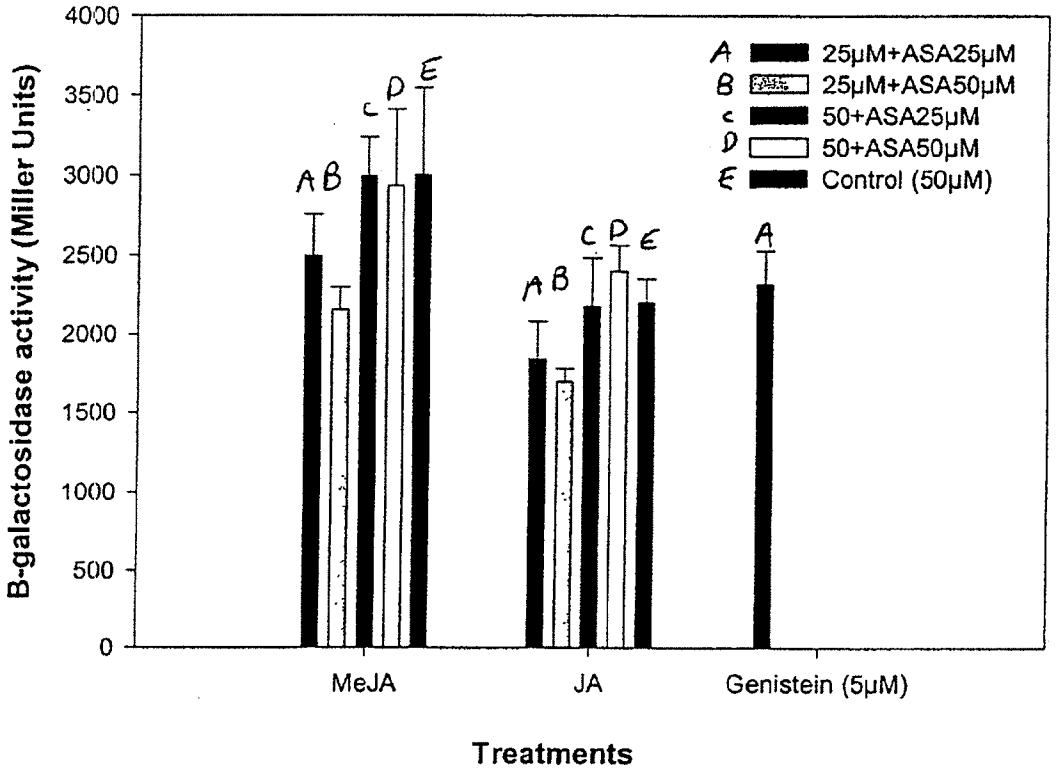

4000
3500
3000
2500
2000
1500
1000
500
0
B-galactosidase activity (Miller Units)
A
B
C
D
E
25μM+ASA25μM
25μM+ASA50μM
50+ASA25μM
50+ASA50μM
Control (50μM)
AB
C
D
E
AB
C
D
E
A
MeJA
JA
Genistein (5μM)
Treatments

Fig. 4

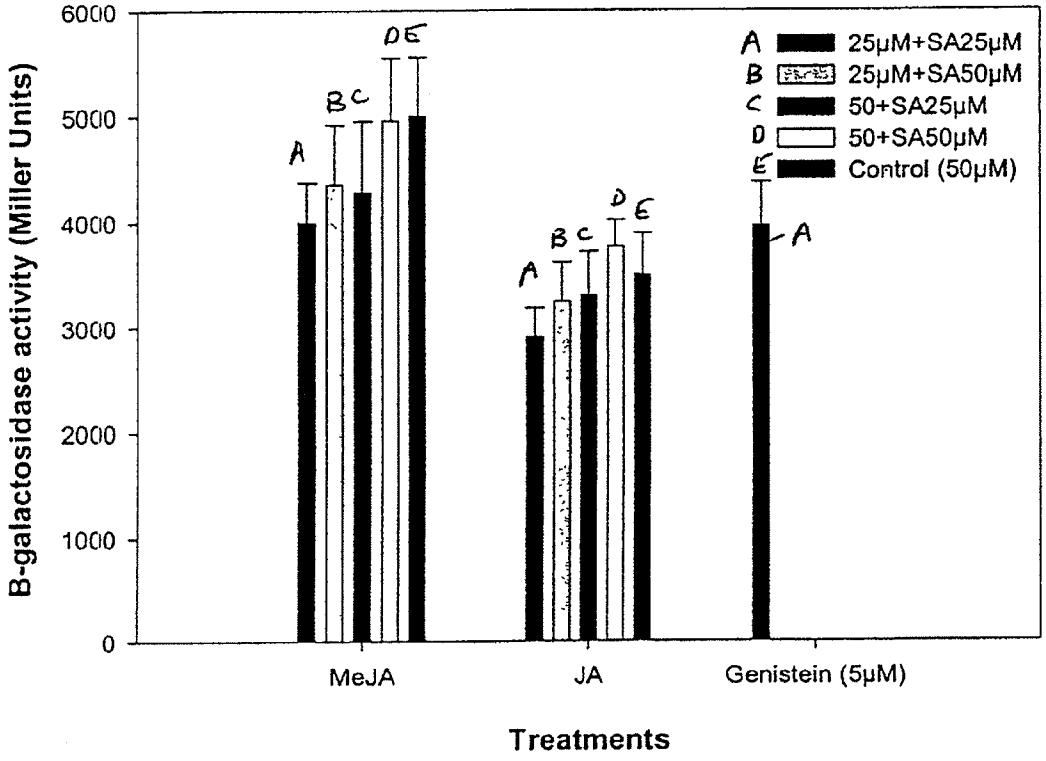

B-galactosidase activity (Miller Units)
6000
5000
4000
3000
2000
1000
0
A
BC
DE
A
BC
DE
A
A 25μM+SA25μM
B 25μM+SA50μM
C 50+SA25μM
D 50+SA50μM
E Control (50μM)
MeJA
JA
Genistein (5μM)
Treatments

Fig. 5

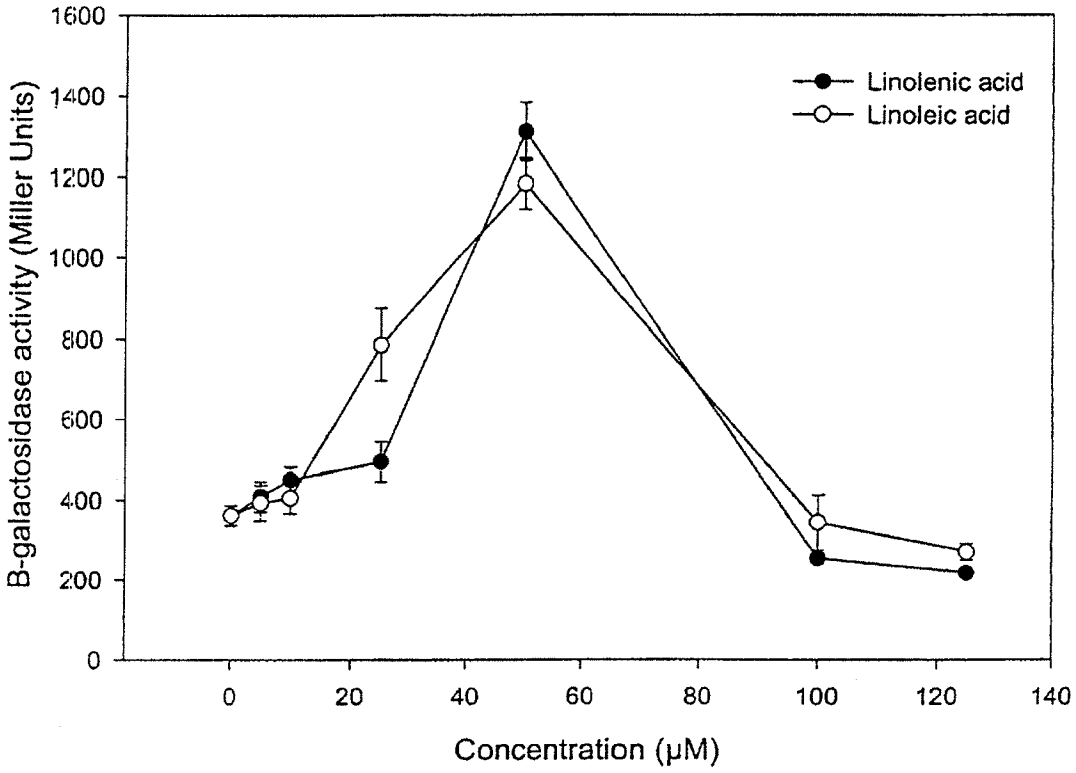

1600
1400
1200
1000
800
600
400
200
0
B-galactosidase activity (Miller Units)
0
20
40
60
80
100
120
140
Concentration (μM)
Linolenic acid
Linoleic acid

Fig. 6

METHODS AND COMPOSITIONS FOR PRODUCTION OF LIPO-CHITO OLIGOSACCHARIDES BY RHIZOBACTERIA

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application Ser. No. 60/303,037, filed Jul. 6, 2001, which is incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to the field of microbiology, more particularly to the production of lipo-chito oligosaccharides by rhizobacteria.

BACKGROUND OF THE INVENTION

The symbiotic relationship between leguminous plants and nitrogen-fixing bacteria involves nodule formation. Nodule formation is a complex process requiring communication between the bacteria and the host plant. During the initial events of symbiosis, plant-to-bacteria signal molecules known as flavonoids are produced by the host plant at low concentrations.

The flavonoids induce the expression of nod genes in the rhizobacteria bacteria. The first level of host specificity in the rhizobacteria-legume interaction is modulated by nodD and its alleles. These regulate the transcription of nodulation genes (nod, nol and noe). The nodD gene product, NodD, acts as a sensor of the plant signal and also regulates transcriptional regulation of the nod genes. The bacterial nod, nol and noe genes are required for infection and nodule formation.

Generally, the transcription of nol/noe genes is induced in the presence of NodD and flavonoids produced by host plants. NodD proteins belong to the LysR family of transcriptional activators. In the presence of flavonoids, NodD proteins bind to a nod box, a conserved promoter sequence preceding the inducible nod genes, and activate the transcription of nod operons.

Expression of the nod genes in the rhizobacteria is believed to be involved in the synthesis of lipo-chito oligosaccharides (LCOs). LCOs are substituted, β1,4 linked trimers, tetramers, and pentamers of N-acetylglucosamine. The LCOs, often described as "nod factors", signal the plant and stimulate the formation of nodules inside the host plants.

Successful colonization of legume plants by nitrogen-fixing rhizobacteria is of significant agricultural and commercial importance. It would be particularly useful to obtain sources of LCOs that could be used to promote nodule formation by rhizobacteria. It would also be of benefit to identify compounds that are useful for inducing nod gene expression in rhizobacteria, resulting in production of LCOs. Rhizobacteria strains that are particularly responsive to nod gene induction, and which produce high levels of LCOs would also be of great utility.

A number of flavonoids which induce nod gene expression in rhizobacteria are known. Isoflavones, primarily genistein and diadzein, are the best inducers of nod::lacZ translational fusions and of the nod YABCUIJ operon in *Bradyrhizobium japonicum*. Genistein ($C_{15}H_{10}O_5$, 5,7,4'-trihydroxyisoflavone, MW 270.2) is a stronger inducer of nod genes in *B. japonicum* than diadzein.

Jasmonic acid (JA) (Chemical Abstracts name: [1R-[1α, 2β(Z)]]-3-oxo-2-(pentenyl)cyclopentaneacetic acid) and its

methyl ester methyl jasmonate (MeJA), are fatty acid derived molecules. They are octadecanoid-based compounds that occur naturally in plants. Jasmonates are involved in plant growth and development, and play an important role in defence responses against pathogens and in wounding responses.

Jasmonic acid is produced in large quantities by the roots of wheat seedlings, and is also produced by fungal microorganisms such as *Botryodiplodia theobromae* and *Gibbrella fujikuroi*, yeast (*Saccharomyces cerevisiae*), and pathogenic and non-pathogenic strains of *Escherichia coli*. Jasmonic acid plays an important role in mycorrhizal signaling and, when applied to an ectomycorrhizal system, has been shown to increase the number of mycorrhized roots, and shoot and root dry weight of spruce seedlings.

Little is known with respect to how jasmonates affect the growth rate of symbiotic microorganisms, or the activation of bacteria-to-plant signaling molecules (nod factors) or their role in host-specific aspects of symbioses when they are present in the rhizosphere. Rosas et al (1998) recently reported that jasmonic acid and methyl jasmonate induced expression of nod genes in *Rhizobium leguminasorum* strain RBL 1284. However, Rosas et al. (1998) did not report whether jasmonic acid or methyl jasmonate increased LCO production as well.

The first step in jasmonic acid biosynthesis is the formation of linoleic acid (Chemical Abstracts name: (Z,Z)-9,12-Octadecadienoic acid) and linolenic acid (Chemical Abstracts name: (Z,Z,Z)-9,12,15-Octadecatrienoic acid) from membrane lipid breakdown, catalysed by phospholipase. Linoleic and linolenic acid are converted to 13-hydroperoxylinolenic acid by the action of lipoxygenase. 13-hydroperoxylinolenic acid is converted into 12,13 expoxy-octadecatrienoic acid in the presence of allene oxide synthase (AOS), and then converted into 12-oxo-phytodienoic acid by allene oxide cyclase (AOC). Following reduction and three steps of β-oxidation, (+)-7-iso-jasmonic acid is formed.

However, despite the role of linoleic and linolenic acid in the biosynthesis of jasmonic acid, it does not appear that they have been considered as possible inducers of nod gene expression or LCO production by rhizobacteria.

Not only is there a need for methods for increasing LCO production by rhizobacteria, there is a need for methods for increasing LCO production by rhizobacteria at low temperatures, in order to improve symbiotic nitrogen fixing symbiosis of rhizobacteria at low temperatures. Optimal symbiotic activity of rhizobacteria in legumes (i.e. nitrogen fixation) often occurs at a temperature far above that at which legume crops are grown. For instance, soybean is a subtropical legume that requires a root zone temperature ("RZT") in the range of about 25 to 30° C. for optimal symbiotic activity. At low temperatures, expression of nod genes in *B. japonicum*, the soybean nitrogen fixing microsymbiont, are inhibited, resulting in a delayed onset of nodulation. Low spring soil temperature is therefore a major factor limiting soybean growth and symbiotic nitrogen fixation in northern regions, such as in Canada. Hence, methods for improving the symbiotic nitrogen fixing activity of rhizobacteria at low temperatures would be of great benefit to legume crop production in cool climates.

SUMMARY OF THE INVENTION

The inventors have discovered that, surprisingly, jasmonic acid, linoleic acid and linolenic acid are useful for inducing LCO production in rhizobacteria. Derivatives of

jasmonic acid, linoleic acid, and linolenic acid, particularly esters, amides, and salts thereof, are also contemplated for use in the present invention. In particular, *Bradyrhizobium japonicum* strain USDA3 is highly responsive to nod gene induction by jasmonic acid esters (jasmonates), resulting in levels of LCO production that are much greater than those obtained from other *B. japonicum* strains. Methyl jasmonate has been determined to be a particularly useful inducer of LCO production in *B. japonicum* strain USDA3.

Thus, in one aspect, the invention provides a method for producing lipo-chito oligosaccharides comprising the steps of culturing rhizobacteria cells in or on a culture medium comprising at least one of: jasmonic acid or a derivative thereof; linoleic acid or a derivative thereof; or, linolenic acid or a derivative thereof; whereby the rhizobacteria cells produce lipo-chito oligosaccharides; and recovering the lipo-chito oligosaccharides from the culture medium.

In another aspect, the invention provides a rhizobial inoculant for promoting nitrogen fixation in legumes at an average root zone temperature below 25° C., the inoculant comprising: (a) rhizobacteria cells; and (b) in an amount sufficient to induce production of lipo-chito oligosaccharides by said rhizobacteria cells, at least one of: jasmonic acid or a derivative thereof; linoleic acid or a derivative thereof; or linolenic acid or a derivative thereof.

In another aspect, the invention provides a method for promoting nitrogen fixation by legumes, comprising inoculating legume plants, seeds, roots, or parts thereof with a rhizobial inoculant as described above, wherein the legume plants are planted under conditions that result in an average daily root zone temperature of less than 25° C.

The invention further provides a kit comprising: a rhizobial inoculant as described above; and instructions for use of the inoculant for inoculating legumes planted under conditions that result in an average daily root zone temperature of less than 25° C.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a graph depicting the effect of various concentrations of genistein, jasmonic acid, methyl jasmonate, salicylic acid, and acetylsalicylic acid on the induction of nod genes in *Bradyrhizobium japonicum* strain USDA3 as measured by β-galactosidase activity (Miller Units).

FIG. **2** is a graph depicting the effect of genistein with the addition of jasmonic acid and methyl jasmonate on the induction of nod genes in *Bradyrhizobium japonicum* strain USDA3 as measured by β-galactosidase activity (Miller Units).

FIG. **3** is a graph depicting the effect of genistein with the addition of salicylic acid and acetylsalicylic acid on the induction of nod genes in *Bradyrhizobium japonicum* strain USDA3 as measured by β-galactosidase activity (Miller Units).

FIG. **4** is a graph depicting the effect of genistein with the addition of methyl jasmonate, jasmonic acid, and salicylic acid on the induction of nod genes in *Bradyrhizobium japonicum* strain USDA3 as measured by β-galactosidase activity (Miller Units).

FIG. **5** is a graph depicting the effect of genistein with the addition of methyl jasmonate, jasmonic acid, and acetylsalicylic acid on the induction of nod genes in *Bradyrhizobium japonicum* strain USDA3 as measured by β-galactosidase activity (Miller Units).

FIG. **6** is a graph depicting the effect of linolenic and linoleic acid on the induction of nod genes in *Bradyrhizobium japonicum* strain USDA3 as measured by β-galactosidase activity (Miller Units).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with one aspect of the invention, LCO production by rhizobacteria species is increased by culturing the rhizobacteria in the presence of jasmonic acid, linoleic acid, linolenic acid, or derivatives thereof.

Any rhizobacteria strain useful for increasing nitrogen fixation in leguminous plants finds application in this aspect of the invention. Rhizobacterial strains of interest are bacteria of the family Rhizobiaciae that are able to enter into a symbiotic nitrogen fixing relationship with a leguminous plant, and supply the plant with nitrogen. Most nitrogen fixing rhizobacteria are members of the genera *Bradyrhizobium, Rhizobium, Sinorhizobium*, and *Azorhizobium*. Many suitable nitrogen fixing rhizobacteria are known to the those of skill in the art, and are available commercially, such as *R. meliloti* and *R. leguminosarum*, and rhizobacteria of the genus *Bradyrhizobium*. Preferred strains include those of the species *Bradyrhizobium japonicum*, particularly strain USDA3, exemplified herein, and available from the United States Department of Agriculture or public culture collections.

Useful derivatives of linoleic acid, linolenic acid, and jasmonic acid include, without limitation, esters, amides, glycosides and salts.

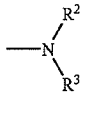
Preferred esters are compounds in which the carboxyl group of linoleic acid, linolenic acid, or jasmonic acid has been replaced with a —COR group, where R is an —OR^1 group,

in which R^1 is:

- an alkyl group, such as a C_1-C_8 unbranched or branched alkyl group, particularly a methyl, ethyl or propyl group;
- an alkenyl group, such as a C_2-C_8 unbranched or branched alkenyl group;
- an alkynyl group, such as a C_2-C_8 unbranched or branched alkynyl group;
- an aryl group having, for example, 6 to 10 carbon atoms; or
- a heteroaryl group having, for example, 4 to 9 carbon atoms, wherein the heteroatoms in the heteroaryl group can be, for example, N, O, P, or S.

Preferred amides are compounds in which the carboxyl group of linoleic acid, linolenic acid, or jasmonic acid has been replaced with a —COR group,

where R is an



group, in which R^2 and R^3 are independently:

- hydrogen;
- an alkyl group, such as a C_1-C_8 unbranched or branched alkyl group, particularly a methyl, ethyl or propyl group;

an alkenyl group, such as a C_2-C_8 unbranched or branched alkenyl group;

an alkynyl group, such as a C_2-C_8 unbranched or branched alkynyl group;

an aryl group having, for example, 6 to 10 carbon atoms; or

a heteroaryl group having, for example, 4 to 9 carbon atoms, wherein the heteroatoms in the heteroaryl group can be, for example, N, O, P, or S.

Esters may be prepared by known methods, such as acid-catalyzed nucleophilic addition, wherein the carboxylic acid is reacted with an alcohol in the presence of a catalytic amount of a mineral acid.

Amides may also be prepared by known methods, such as by reacting the carboxylic acid with the appropriate amine in the presence of a coupling agent such as dicyclohexyl carbodiimide (DCC), under neutral conditions.

Suitable salts of linoleic acid, linolenic acid, and jasmonic acid include e.g. base addition salts. The bases that may be used as reagents to prepare metabolically acceptable base salts of these compounds include, but are not limited to those derived from cations such as alkali metal cations (e.g., potassium and sodium) and alkaline earth metal cations (e.g., calcium and magnesium).

These salts may be readily prepared by mixing together a solution of linoleic acid, linolenic acid, or jasmonic acid with a solution of the base. The salt may be precipitated from solution and be collected by filtration or may be recovered by other means such as by evaporation of the solvent.

Jasmonic acid can be used in this invention as a racemic mixture (i.e. as a mixture containing approximately equal amounts of each of its enantiomers), or as an enantiomerically enriched mixture, in which one of its enantiomers is present in excess over the other. In a preferred embodiment, jasmonic acid is used as an enantiomerically enriched mixture containing at least 90% by weight of one of its enantiomers, and 10% by weight or less of the other of its enantiomers. More preferably, jasmonic acid is used as an enantiomerically enriched mixture containing at least 99% by weight of one of its enantiomers, and 1% or less of the other of its enantiomers. Even more preferably, jasmonic acid is used in enatiomerically pure form, i.e., as 100% by weight of one of its enantiomers.

In order to produce LCOs by the methods of the invention, the rhizobacteria cells are cultured in or on a culture medium containing the inducing compound. Suitable culture media and culture conditions are known in the art. For instance, yeast extract mannitol (YEM) medium, as exemplified herein, may be used. In order to obtain large quantities of LCOs, the rhizobacteria are preferably cultured in large-scale continuous liquid fermentation systems as are known and commercially available. Other culture conditions, such as aeration, agitation, temperature, etc. are not critical to the invention, and suitable culture conditions for growing rhizobacteria are known in the art.

The jasmonic acid, linoleic acid, linolenic acid or derivative thereof, or a combination of two or more thereof, is present in the culture medium at a concentration preferably in the range of about 10 to about 200 μM, more preferably about 25 to about 100 μM, and even more preferably about 50 μM.

Genistein may additionally be present in the culture medium at a concentration preferably in the range of about 1 to about 100 μm, more preferably about 1 to about 25 μM, and even more preferably about 5 μM.

As used herein, the term "about" means up to ±25% of the stated value. For instance, "about 50 μM" encompasses the range of 37.5 μM to 62.5 μM i.e. 50 μm ±25%.

Methyl jasmonate is a preferred inducing compound. In a particularly preferred embodiment, methyl jasmonate and genistein are both present in the culture medium.

The LCOs may be recovered from the culture medium by any suitable technique, the choice of which is not critical to the invention. For instance, high-performance liquid chromatography (HPLC), as exemplified in Example 1 herein, may be used.

Rhizobial inoculant compositions containing linoleic acid, linolenic acid, jasmonic acid, or derivatives thereof, or genistein, in amounts sufficient to induce LCO production by the rhizobacteria, and which are suitable for use for inoculating legume plants to promote nitrogen fixation, may be formulated in accordance with known techniques. Techniques for formulating inoculants are known in the art. Typically, inoculants are in a dried or liquid form. Dried inoculants (powdered peat inoculants) generally contain dried bacteria mixed with sterilized peat and then packaged. The inoculant may contain carriers, blending agents, extenders, excipients, adjuvants, et cetera, as are known in the art. The dried inoculant may also include a binding or sticking agent to help the bacteria adhere to the plant seeds, roots, etc. upon application. Liquid inoculants may be prepared by suspending the bacteria in a suitable diluent or carrier, such as water.

In an alternative embodiment, the rhizobial inoculant may not contain jasmonic acid, linoleic acid, linolenic acid or a derivative thereof. Instead, the rhizobacteria will previously have been grown in or on a culture medium containing jasmonic acid, linoleic acid, linolenic acid or a derivative thereof, whereby the beneficial effects of such compounds on nod gene induction or LCO production is obtained, and the rhizobacteria so produced are then formulated into a rhizobial inoculant.

The rhizobial inoculants of the invention are useful for increasing nitrogen fixation in all leguminous plants in which nitrogen fixation by rhizobacteria occurs. Non-limiting examples of legumes include soybeans, peanuts, all the pulses, including peas and lentils, all the beans, and major forage crops, such as alfalfa and clover. Legumes also include many more plants of lesser agricultural importance, such as lupines, sainfoin, trefoil, and even some small tree species.

Techniques for applying rhizobial inoculants to legumes are known in the art, including appropriate modes of administration, frequency of administration, dosages, et cetera. Typically, liquid or powdered compositions are applied to seeds, although the rhizobacteria composition may also be applied to soil, either before or after planting, or contemporaneously therewith. Any part of the plant may be inoculated, such as the roots, seeds, stems or leaves.

Inoculant compositions according to the invention are preferably applied to plants grown under conditions that result in an average daily root zone temperature of less than 25°, more preferably less than 20° C., 19° C., 18° C., 17° C., 16° C., 15° C., 14° C., 13° C., 12° C., 11° C. or 10° C., as may be found in many northerly climates. Average daily root zone temperature is calculated as the average 24-hour root zone temperature of the plant over the entire growing season, from the time of planting through to harvest. Alternatively, and where specifically indicated herein, average daily root zone temperature is calculated as the average

24-hour root zone temperature for a particular month, e.g. March, April, May, June, July, August, September or October.

Kits containing inoculants of the invention will typically include one or more containers of the inoculant, and printed instructions for using the inoculant for promoting plant growth. The kit may also include tools or instruments for reconstituting, measuring, mixing, or applying the inoculant, and will vary in accordance with the particular formulation and intended use of the inoculant.

Further details concerning the preparation of bacterial inoculants and methods for inoculating plants with bacterial inoculants are found in e.g. U.S. Pat. Nos. 5,586,411; 5,697,186; 5,484,464; 5,906,929; 5,288,296; 4,875,921; 4,828,600; 5,951,978; 5,183,759; 5,041,383; 6,077,505; 5,916,029; 5,360,606; 5,292,507; 5,229,114; 4,421,544; and 4,367,609, each of which is incorporated herein by reference.

Unless defined otherwise, all technical and scientific terms used herein are intended to have the same meaning as is commonly understood by one of ordinary skill in the relevant art.

As used herein, the singular forms "a," "an", and "the" include the plural reference unless the context clearly dictates otherwise.

The following examples are illustrative, but not limiting, of the present invention. Other suitable modifications and adaptations are of the variety normally encountered by those skilled in the art and are fully within the spirit and scope of the present invention.

EXAMPLE 1

Experiment 1

This experiment evaluated the effect of salicylates and fatty acids including jasmonic acid and its methyl ester, methyl jasmonate, and on the induction of nod genes in *Bradyrhizobium japonicum* strain USDA3.

Bacterial Strains

Bradyrhizobium japonicum strain USDA3, harboring plasmid GG4, was used in the experiment. Plasmid GG4 contains a translational fusion between *B. japonicum* nod Y open-reading frame and lac Z of *Escherichia coli*. Hence, the nod gene expression activity due to various inducers was indirectly measured by the amount of β-galactosidase activity.

Bacterial Growth and Incubation

Bacteria were grown in yeast extract mannitol (YEM) medium (mannitol 10.0 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, and yeast extract 0.4 g dissolved in 1000 mL of dd H_2O). In order to ensure the maintenance of the strains, tetracycline was added to the bacterial cultures at a concentration of 20 mg l^{-1}. One hundred mL cultures of each strain, in 250 mL flasks, were shaken at 150 rev min^{-1} at 30° C. for 3-5 days and then subcultured into 400 mL of fresh medium in 1000 mL flasks. The cultures were incubated until the OD_{620} reached 0.2-0.3. The cultures were then divided into 5 mL tubes (sterilized).

Stock solutions of various inducers: genistein, salicylic acid and acetylsalicylic acid made in methanol and linolenic, linoleic, and jasmonic acid and methyl jasmonate were made in ethanol, and an appropriate amount of these inducers was added into the tubes before addition of bacterial culture. In the tubes, the final concentration of all the inducers was maintained as 5, 10, 25, 50 and 100 μM. Tubes were collected after 18 h of incubation and stored at −20° C. until the β-galactosidase activity was measured. Genistein (4',5,7, trihydroxyisoflavone, purity 98%,), linolenic acid (9,12,15-Octadecatrienoic acid, purity 99%), linoleic acid (cis-9, cis-12-Octadecadienoic acid, purity 99%), jasmonic acid (±Jasmonic acid, $C_{12}H_{18}O_3$) and salicylic acid (2-hydroxybenzoic acid, purity 99.0%) were obtained from Sigma, while acetylsalicylic acid ($C_9H_8O_4$, 99% purity) was obtained from Acros and methyl jasmonate (95% purity) was obtained from Aldrich Chemical Company, Inc.

β-Galactosidase Activity Assay

β-galactosidase is an enzyme which hydrolyzes β-D-galactosides. The activity of this enzyme can easily be measured with colorless substrates, which upon hydrolysis produce colored products. O-nitrophenyl-β-galactoside (ONPG) is a colorless compound and is converted to galactose and O-nitrophenol in the presence of β-galactosidase. The O-nitrophenol is yellow and can be measured by its absorption at 420 nm. In the present study, nod gene expression was indirectly measured by β-galactosidase activity following the method of Miller (1972) as modified by Stachel et al, (1985). Briefly, 0.5 mL culture was mixed with 2×Z buffer ($Na_2HPO_4.7H_2O$, 16.1 g, $NaH_2PO_4.H_2O$, 5.5 g, KCl 0.75 g, $MgSO_4.7H2O$ 0.246 g, β-mercaptoethanol 2.7 g, pH 7.0 dissolved in 500 mL water) and 40 μL toluene and vortexed for 10 seconds. The culture was then incubated in a water bath at 37° C. for 30 minutes and then 0.2 mL of O-nitrophenyl β-D-galactopyranoside (ONPG) was added at a concentration of 4 mg mL^{-1}. The cultures were incubated again in the water bath until the color of the culture changed. The reaction time was recorded and the reaction was stopped with 1M Na_2CO_3. After centrifugation at 10,000 rpm for 4 minutes, spectrophotometric reading at OD_{420} and OD_{600} were taken and the β-galactosidase activity was measured (β-galactosidase activity=OD_{420}*1000/OD_{600}*T*V, where T is the reaction time and V is the volume of the bacterial culture used for enzyme assay).

Statistical Analysis

The experiment was designed in a randomized complete block design (RCBD) with three replicates. The experiment was run two times and the results were similar in both the cases. Data are shown from the second experiment. Statistical analysis of the data was done with analysis of variance using the Statistical Analysis System computer package (SAS Institute, 1988). Comparisons among treatment means were made with an ANOVA protected LSD at the 0.05 level of statistical significance.

Experiment 2

This experiment was conducted to evaluate the effect of jasmonates on the production of lipo-chito oligosaccharides (LCOs) from *Bradyrhizobium japonicum* USDA 3.

Bacterial Culture and Incubation

Bradyrhizobium japonicum (strain USDA 3) was grown at 28° C. in yeast extract mannitol medium (YEM) (mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, yeast extract 0.4 g, and distilled water 1000 ml), pH 6.8, shaken at 150 rpm until the OD_{620} reached 0.4-0.6 (4-6 d) in the dark. Thereafter, 2.0 l of bacterial subculture was started by inoculating with material from the first culture (5 ml of the first culture per 250 ml of YEM media), until the OD_{620} reached to 0.2-0.4. At this stage, methyl jasmonate, jasmonic acid and genistein were added to culture so that the final concentraton for methyl jasmonate and jasmonic acid was 5, 10, 25, 50 and 100 μM and 5 μM for genistein. The culture was incubated for 48-96 h and the LCO extracted according to the following procedure.

Extraction and Purification of LCO

The LCO from the induced bacterial culture was isolated with XAD resins. Before using XAD resins for extraction, they were first conditioned. Each 40 grams of XAD resins were first washed two times with 20 mL acetone after conditioning for 15 minutes. This was followed by two washes of 20 mL methanol when the resins were conditioned for 15 minutes. Finally, the XAD resins were conditioned (5 min) and washed with 20 mL dd. water two times. The conditioned resins were placed in the refrigerator at 4° C. until use.

For each one liter of bacterial culture, 40 grams of resin was added to the flask and these were shaken together overnight at 150 rpm. The culture along with the resin was poured through a funnel into a flask fitted with a course brass mesh (fine enough to allow the culture go quickly through and filter out the resin without any loss). At this stage, the resins are washed with water. In order to extract LCO from the resin, the XAD resin was passed through a vacuum filtration system. The fritted glass base of the system was fitted with Whatman #1 paper disk with the objective to keep the solvent with the bead until vacuum is applied. The resin beads were washed two times with 40 mL methanol after conditioning for 5 minutes. After this, the beads were again washed two times with 30 mL acetone after conditioning for 15 minutes. The filtrate of the methanol and acetone washes was collected and transferred to a 250 mL boiling flask and placed on a rotary evaporator (Yamota RE500, Yamato, USA) operated at 45° C. and a speed of 125 rpm. Evaporation was continued until the flask became dry. The extract was then resuspended in 4 ml of 18% acetonitrile and kept in the dark at 4° C. in a sealed glass vial, until use.

HPLC (equipped with Waters Model 510 HPLC pump, Waters model 712 WISP and Waters model 410 differential refractometer, Waters, Mass., USA) analysis was conducted with a Vydac C18 reversed-phase column (Vydac, Calif., USA; catalogue no. 218TP54) with a flow rate of 1.0 ml min^{-1} and a Vydac guard column (catalogue no. 218GK54). As a baseline 18% acetonitrile (AcN/H_2O; w/w) was run through the system for at least 10 min. prior to sample injection. The sample was loaded and isocratic elution was conducted with 18% of AcN for 45 min to remove all non-polar light fractions. Thereafter, gradient elution was conducted for 90 min with 18-82% AcN. The LCO eluted at 94-96 min of HPLC run time.

Biological Activity of LCO (Root Hair Deformation Assay)

Biological activity of a compound is an important step to confirm its activity. LCOs are signal molecules that induce root hair curling in host plants. We tested the biological activity of MeJA induced LCO in soybean roots and found root hair curling (HAC) and deformation (HAD) in soybean root segments in a fashion similar to that demonstrated by genistein induced LCO production. Root hair deformation was studied according to the procedure of Prithiviraj et al (2000). Seeds of soybean (cultivars: AC Bravor, OAC Brussels, Maple Glen, Nordet, cv. 9007) were surface-sterilized with 2% sodium hypochlorite for 2 min. followed by four washes of sterile distilled water. The seeds were then placed on 1.5% water agar (20 ml) in 9 cm diameter Petri dishes (two seeds per plate). The Petri dishes were incubated in the dark at 25° C. for 7-8 d; so that the seeds germinated and developed tap and lateral roots on the agar surface. Lateral roots with abundant root hairs were excised and placed on sterile grease-free glass slides containing 40-60 μl of LCO solution. The slides were incubated in a closed moist chamber at 25° C. in the dark and after 24 h of incubation time, the slides were removed, the roots were fixed in a staining solution [methylene blue (0.02% w/v)+glycerol (20% v/v)+phenol (10% w/v)]. Light microscopic studies were observed for root hair deformation of the jasmonic acid, methyl jasmonate and genistein induced LCO. Each treatment had three replicate lateral roots, and a minimum of 100 root hairs was observed from each replicate.

Results

Genistein and diadzein are produced naturally from legume roots and induce the expression of nod genes in *Bradyrhizobium japonicum*. Here we studied the possible effect of various other compounds, salicylates (salicylic and acetylsalicylic acid) and fatty acids (linolenic and linoleic acid) including jasmonic acid and its methyl ester, methyl jasmonate on the induction of nod genes in *B. japonicum* USDA3. Genistein, the natural inducer of nod genes, was used a positive control since genistein is a stronger inducer of nod gene in *B. japonicum* than diadzein.

Our results with β-galactosidase assay showed that jasmonic acid (JA) and methyl jasmonate (MeJA) strongly induced the expression of nod genes at concentrations from 25 to 100 μM (FIG. **1**). However, methyl jasmonate showed maximum induction of nod genes, as compared to jasmonic acid, at the optimum genistein concentration (5 μM). At concentrations 10 μM or less, jasmonic acid as well as methyl jasmonate showed no activity (FIG. **1**).

Linolenic and linoleic acid also induced the expression of nod genes with 50 μM as the optimum concentration (FIG. **6**). However, nod gene induction due to linolenic and linoleic acid, at their optimum concentrations, was lower than the optimum JA and MeJA concentrations.

On the other hand, salicylates (salicylic acid and acetylsalicylic acid) did not show any activity on the induction of nod genes; the β-galactosidase activity was not different from the control (without any inducer) at all concentrations (FIG. **1**).

In order to study the possible interactions among genistein and various other compounds, various combinations of jasmonic acid, methyl jasmonate, salicylic acid, acetylsalicylic acid and genistein were tested and the β-galactosidase activity estimated. The optimum level of genistein (5 μM) and equimolar combinations of JA, MeJA, SA and ASA at 25 and 50 μM concentrations were used.

The results showed that simultaneous addition of genistein with either JA or MeJA at various concentrations did not result in any synergistic effect when β-galactosidase activity was measured (FIG. **2**). SA and ASA did not show any activity alone, and in order to study their interaction with other inducers, both SA and ASA were tested at two concentrations (25 and 50 μM) with JA, MeJA and genistein (at 5 μM, the optimum level for nod gene induction by genistein).

In all the combinations studied, both SA and ASA did not show inhibitory or inducing activity, when analyzed by β-galactosidase assay (FIGS. **3**, **4**, and **5**).

The β-galactosidase assay indirectly measures the transcription of nod genes, and in order to measure the end product of nod gene expression [lipo-chito oligosaccharides—LCOs], wild type USDA3 strain were induced with JA, MeJA and genistein (as a positive control) and LCO was isolated. Both JA and MeJA, at optimum concentrations, induced the production of LCOs. MeJA induced *B. japonicum* to produce more LCO and at the 100 μM concentration, MeJA induced substantially more LCO than the optimum genistein concentration (5 μM).

EXAMPLE 2

This experiment was conducted to evaluate nod gene induction by jasmonates in various *Bradyrhizobium japonicum* strains.

The effect of 0-100 μM concentrations of each of acetylsalicylic acid, salicylic acid, jasmonic acid, methyl jasmonate, and genistein, on induction of nod gene expression in *Bradyrhizobium japonicum* strains USDA31, USDA76, USDA121, and USDA3, was evaluated in accordance with the β-galactosidase assay of Experiment 1 in Example 1 above.

As shown in Table 1, induction of nod gene expression by jasmonic acid and methyl jasmonate was much greater in strain USDA3 than in the other strains tested, particularly at concentrations of jasmonic acid or methyl jasmonate between 25-100 μM. Moreover, methyl jasmonate had a substantially greater inducing effect than jasmonic acid.

TABLE 1

Differential nod gene induction response of *Bradyrhizobium japoniucm* strains to jasmonates

Conc. (μM)	ASA	SA	JA	MeJA	GEN
USDA31 (GG1)					
0	522.6 ± 15.9	522.6 ± 15.9	522.6 ± 15.9	522.6 ± 15.9	522.6 ± 15.9
5	636.5 ± 66.9	587 ± 66.6	1653.3 ± 105.2	472.2 ± 14.8	2283.8 ± 57.7
10	563.3 ± 37.9	536.4 ± 40.1	2079 ± 254.9	485.3 ± 42.5	2007.6 ± 36.7
25	719.5 ± 75.2	522.4 ± 65.3	1853.3 ± 314.5	403.3 ± 5.6	1821 ± 89.2
50	676.6 ± 85.8	476.1 ± 28.4	1884.3 ± 289.3	500.1 ± 32	1121.1 ± 156.9
100	384.8 ± 34.4	519.3 ± 43.5	796 ± 67.3	392.9 ± 22.7	491.9 ± 31.6
USDA 76 (GG2)					
0	411.8 ± 26.8	411.8 ± 26.8	411.8 ± 26.8	411.8 ± 26.8	411.8 ± 26.8
5	363.2 ± 34.1	562.2 ± 26.6	393.5 ± 30.5	435.4 ± 39	1421.7 ± 93.4
10	341 ± 21.1	411.8 ± 75.3	447.9 ± 21	418.4 ± 53.1	1320.8 ± 75.99
25	418.8 ± 59.5	473 ± 58.3	420.1 ± 4.6	392.7 ± 2.1	1383.8 ± 98.22
50	376.2 ± 37.2	464.6 ± 36.2	375.1 ± 35.3	410.4 ± 24.5	1160.7 ± 89.7
100	413.1 ± 85.5	430.7 ± 55.3	357.7 ± 9.7	386.4 ± 37.3	413.7 ± 7.4
USDA121 (GG3)					
0	476.8 ± 86.6	476.8 ± 86.6	476.8 ± 86.6	476.8 ± 86.6	476.8 ± 86.6
5	422.6 ± 64.9	404.1 ± 62.5	540.3 ± 79.3	518 ± 42.6	2081.7 ± 148.5
10	425 ± 39.4	472.7 ± 54.3	473.7 ± 58.3	487.1 ± 33	1685.1 ± 53.3
25	479.9 ± 88.6	435.8 ± 69.2	496 ± 49.2	427 ± 29.4	1286.8 ± 145.5
50	445.4 ± 99	434.2 ± 26.3	412.7 ± 22.9	549.2 ± 81.6	1154.5 ± 121.1
100	325.6 ± 58.8	369.8 ± 114.9	329.5 ± 19.3	487.9 ± 89.3	430.9 ± 80.6
USDA3 (GG4)					
0	362 ± 25	362 ± 25	362 ± 25	362 ± 25	362 ± 25
5	396 ± 14.7	398 ± 13	368 ± 23	448 ± 30	3304 ± 333
10	372 ± 16	402 ± 33	362 ± 9	500 ± 61	2500 ± 94
25	382 ± 16	414 ± 17	3200 ± 230	3000 ± 350	2300 ± 219
50	464 ± 7	435 ± 26	3900 ± 150	4700 ± 450	1930 ± 74
100	527 ± 36	515 ± 583	550 ± 210	4500 ± 400	406 ± 15

Legends:
ASA Acetylsalicylic acid
SA Salicylic acid
JA Jasmonic acid
MeJA Methyl jasmonate

EXAMPLE 3

This example illustrates that jasmonic acid (JA) can prevent the inhibition of LCO production under low temperature, and that JA enhances the ability of genistein to induce LCO production.

Cultures of *B. japonicum* (strain USDA 110) were grown at 28° C. in 600 ml of sterile yeast mannitol medium (YEM) at pH 6.8. The culture was shaken at 150 rpm until an OD_{620} of 0.4 to 0.6 was achieved (4 to 6 days). When the culture reached this stage, it was divided equally into four flasks (150 mL) and specific concentrations of JA and genistein were added to the cultures.

One hundred and fifty mL of each culture were extracted with 40 mL of the HPLC-grade butanol (Fisher Science Ltd., 112 Colonnade Road, Nepean, ON, Canada) by shaking together for 5 minutes. The two phases were then allowed to separate by leaving the mixture to stand overnight. The upper layer (butanol) was collected with a glass pipette and 25 mL were placed in a 250 ml evaporation flask. LCO extract solutions were stored at 4° C. until evaporation. This step was conducted at 80° C. with a Yamato RE 500 Rotary Evaporator (Yamato Scientific American Inc., Orangeburge, N.Y., USA). The butanol phase was evaporated down to 2 to 3 mL. The resulting light brown material was dissolved in 4 ml of 18% acetonitrile and stored in the dark at 4° C. in a glass tube sealed with parafilm.

For HPLC analysis we used a Vydac C18 reversed—phase column (VYDAC, Hesperia, Calif., USA) with a flow rate of 1.0 mL/min and a Vydac guard column (VYDAC, Hesperia, Calif., USA). We used at least 10 min. of isocratic run with 18% acetonitrile (AcN/H_2O; w/w) to establish a baseline. The baseline value was always about 0.010. When a sample was loaded we conducted an isocratic elution with 18% AcN for 45 min. This step removed all non-polar light fractions. We then applied a gradient elution (18 to 82% AcN) for 70 min. LCOs begin to appear at 84 to 86 min of

HPLC run time. We used *B. japonicum* Nod factor (BjV ($C_{18:1}$, MeFuc)) obtained from G. Stacey (University of Tennessee, Knoxville, Tenn.) as a standard. The identity of the LCO was confirmed by the Complex Carbohydrate Research Center at the University of Georgia with FAB-MS and MALDI-TOF spectrometry analysis.

There were two experiments conducted. In the first the treatments consisted of factorial combinations of two concentrations (0 and 20 μM) of two signal materials (JA and genistein (zhang and Smith, 1995)). The experiment was organized following a randomized complete block design with three blocks. In experiment 1, two temperatures were used (17 and 25° C.). The LCO was extracted 10 h after the addition of the signal compounds. Experiment 2 was generally like experiment one except that only one temperature, 17° C. was used and LCO was extracted at 5, 10, 20 hour after JA and genistein were added.

Each of the above experiments was conducted two times and the experimental results were pooled and analyzed statistically by analysis of variance using the Statistical Analysis System computer package (SAS Institute Inc. 1980). A least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 level of significance, when analysis of variance showed significant treatment effects. Since the effects of temperature on growth were very large, the data from two temperatures used in the first experiment were analyzed separately.

Our data showed that JA can improve LCO production at a low temperature (17° C.) but not at 25° C. However, JA alone can not promote LCO production by *B. japonicum* USDA 110 at either temperature; genistein must be present (Table 2). There was no statistical interaction between levels of the JA and genistein factors.

We suppose that at low temperatures *B. japonicum* needs additional signal compounds, which come in the form JA. When JA was supplied in addition to genistein, it helped to prevent the low temperature inhibition of *B. japonicum* LCO production. Previous studies suggest that under at least some stresses (low temperature (17° C.) and pathogen infection) and for higher levels of nodulation under low temperature stress, the biosynthesis of JA in plants is a necessary response. But under optimum temperature conditions the biosynthesis of JA may not be required for soybean nodulation because we found that JA did not improve LCO production by *B. japonicum* under these conditions. At low temperatures it is probable that some gene is activated by application of JA and produces a product that improves the action of genistein on LCO production. The stimulation of LCO production by JA is important because we found that higher concentrations of genistein inhibit LCO production in our previous experiments (unpublished data) placing an upper limit on the amount of improvement in inoculum efficacy that can be achieved by genistein addition.

Our previous experimental work (unpublished data) and the findings reported here indicate that the peak-time for LCO production is around 8-10 hours after the genistein was added to the cell culture (Table 3). It is interesting to note that stimulation of LCO production by JA was apparent after 5 to 10 h. We did not find any difference in LCO production between JA at 20 μM plus genistein at 0 μM and JA at 20 μM and genistein at 20 μM at 20 hour (Table 3). We do not know why LCO production by *B. japonicum* at 20 h is less than at 10 h with genistein and JA, but it may be that the soybean—*B. japonicum* system does not need additional LCO and degrades it, or that The LCO has been changed to other compounds involved in on, reducing the LCO content.

In summary, we found that JA can help genistein promote LCO production by *B. japonicum* USDA 110 under low temperature, but JA cannot initiate LCO production in the absence of genistein.

TABLE 2

The effects of jasmonic acid and genistein concentration on LCO production by *B. japonicum* USDA 110 at 17 and 25° C. **, NS indicates differences at the 0.05 probability level, and not significant at P < 0.11 respectively.

Factors		LCO production (μg cell-1)	
JA concentration (μM)	Genistein concentration (μM)	17° C.	25° C.
0	0	0	0
0	20	236	439
20	0	0	0
20	20	321	451
$LSD_{0.05}$		78	152
JA		**	NS
Genistein		**	**
JA × Genistein		NS	NS

TABLE 3

The effects of jasmonic acid and genistein concentration on the change of LCO production by *B. japonicum* USDA 110 with the time of addition to the culture. **, NS indicates differences at the 0.05 probability level.

Factors				
JA concentration (μM)	Genistein concentration (μM)	LCO production (μg cell-1) 5 hour	10 hour	20 hour
0	0	0	0	0
0	20	113	493	88
20	0	0	0	0
20	20	201	1046	81
$LSD_{0.05}$		57	268	39
JA		**	**	NS
Genistein		**	**	**
JA × Genistein		NS	NS	NS

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All patents and publications cited herein are hereby fully incorporated by reference in their entirety, and are illustrative of the level of skill in the art to which this invention pertains. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Having now fully described this invention, it will be understood to those of ordinary skill in the art that the methods of the present invention can be carried out with a wide and equivalent range of conditions, formulations, and other parameters without departing from the scope of the invention or any embodiments thereof.

The invention claimed is:

1. A method for promoting nitrogen fixation by soybean plants, comprising:

inoculating a soybean plant with a rhizobial inoculant, wherein said rhizobial inoculant comprises rhizobacteria of the species *Bradyrhizobium japonicum*, and at least one compound selected from the group consisting of: jasmonic acid or an ester or salt thereof; linoleic acid or an ester or salt thereof; and linolenic acid or an ester or salt thereof.

2. The method according to claim **1**, wherein said inoculating comprises applying said rhizobial inoculant to said plant, or to a part, seed or root thereof, or to soil adjacent thereto.

3. The method according to claim **2**, wherein said inoculating comprises applying said rhizobial inoculant to said soil prior to planting.

4. The method according to claim **1**, further comprising growing said plants under conditions that result in an average daily root zone temperature of less than 25° C.

5. The method according to claim **1**, wherein said at least one compound comprises jasmonic acid or an ester or salt thereof.

6. The method according to claim **1**, wherein said at least one compound comprises an ester of jasmonic acid.

7. The method according to claim **1**, wherein said at least one compound comprises methyl jasmonate.

8. The method according to claim **1**, wherein said at least one compound comprises jasmonic acid.

9. The method according to claim **4**, wherein said average daily root zone temperature is less than 20° C.

10. The method according to claim **4**, wherein said average daily root zone temperature is less than 17° C.

11. The method according to claim **4**, wherein said average daily root zone temperature is less than 15° C.

12. The method according to claim **4**, wherein said average daily root zone temperature is less than 13° C.

13. The method according to claim **4**, wherein said average daily root zone temperature is less than 10° C.

14. The method according to claim **1**, wherein said rhizobial inoculant is in a dried or liquid form.

15. The method according to claim **1**, wherein said rhizobial inoculant comprises a carrier, blending agent, extender, excipient, or adjuvant.

16. A rhizobial inoculant for promoting nitrogen fixation by soybean plants, said rhizobial inoculant comprising rhizobacteria of the species *Bradyrhizobium japonicum*, and at least one compound selected from the group consisting of: jasmonic acid or an ester or salt thereof; linoleic acid or an ester or salt thereof; and linolenic acid or an ester or salt thereof.

17. The rhizobial inoculant according to claim **16**, wherein said at least one compound comprises jasmonic acid or an ester, or salt thereof.

18. The rhizobial inoculant according to claim **16**, wherein said at least one compound comprises jasmonic acid.

19. The rhizobial inoculant according to claim **16**, wherein said at least one compound comprises an ester of jasmonic acid.

20. The rhizobial inoculant according to claim **16**, wherein said at least at least one compound comprises methyl jasmonate.

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